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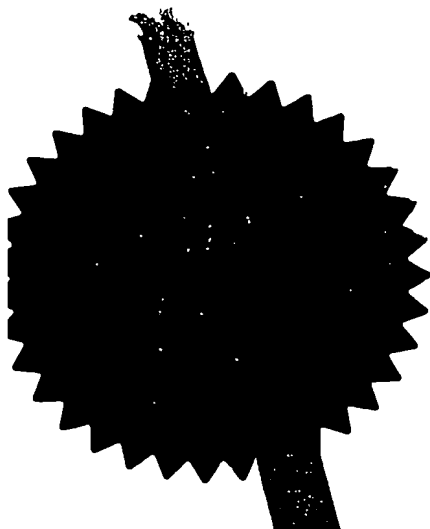
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CASE NO. 39

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08571242001

Patents ADP number (if you know it)

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4. Title of the invention

SUBSTANCES

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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Claim(s) 12 (TWELVE)

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SUBSTANCES

The present invention relates to proteinaceous particles, for example phage or ribosome particles, displaying T cell receptors (TCRs).

5

Background to the Invention

Native TCRs

As is described in, for example, WO 99/60120 TCRs mediate the recognition of specific Major Histocompatibility Complex (MHC)-peptide complexes by T cells and, as such, are essential to the functioning of the cellular arm of the immune system.

10

Antibodies and TCRs are the only two types of molecules which recognise antigens in a specific manner, and thus the TCR is the only receptor for particular peptide antigens presented in MHC, the alien peptide often being the only sign of an abnormality within a cell. T cell recognition occurs when a T-cell and an antigen presenting cell (APC) are in direct physical contact, and is initiated by ligation of antigen-specific TCRs with pMHC complexes.

15

The native TCR is a heterodimeric cell surface protein of the immunoglobulin superfamily which is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. TCRs exist in $\alpha\beta$ and $\gamma\delta$ forms, which are structurally similar but have quite distinct anatomical locations and probably functions. The MHC class I and class II ligands are also immunoglobulin superfamily proteins but are specialised for antigen presentation, with a highly polymorphic peptide binding site which enables them to present a diverse array of short peptide fragments at the APC cell surface.

20

25

Two further classes of proteins are known to be capable of functioning as TCR ligands. (1) CD1 antigens are MHC class I-related molecules whose genes are located on a different chromosome from the classical MHC class I and class II antigens. CD1 molecules are capable of presenting peptide and non-peptide (eg lipid, glycolipid)

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moieties to T cells in a manner analogous to conventional class I and class II-MHC-pep complexes. See, for example (Barclay et al, (1997) *The Leucocyte Antigen Factsbook* 2nd Edition, Academic Press) and (Bauer (1997) *Eur J Immunol* 27 (6) 1366-1373)) (2) Bacterial superantigens are soluble toxins which are capable of binding both class II MHC molecules and a subset of TCRs. (Fraser (1989) *Nature* 339 221-223) Many superantigens exhibit specificity for one or two V β segments, whereas others exhibit more promiscuous binding. In any event, superantigens are capable of eliciting an enhanced immune response by virtue of their ability to stimulate subsets of T cells in a polyclonal fashion.

The extracellular portion of native heterodimeric $\alpha\beta$ and $\gamma\delta$ TCRs consist of two polypeptides each of which has a membrane-proximal constant domain, and a membrane-distal variable domain. Each of the constant and variable domains includes an intra-chain disulfide bond. The variable domains contain the highly polymorphic loops analogous to the complementarity determining regions (CDRs) of antibodies. CDR3 of $\alpha\beta$ TCRs interact with the peptide presented by MHC, and CDRs 1 and 2 of $\alpha\beta$ TCRs interact with the peptide and the MHC. The diversity of TCR sequences is generated via somatic rearrangement of linked variable (V), diversity (D), joining (J), and constant genes

Functional α and γ chain polypeptides are formed by rearranged V-J-C regions, whereas β and δ chains consist of V-D-J-C regions. The extracellular constant domain has a membrane proximal region and an immunoglobulin region. There are single α and δ chain constant domains, known as TRAC and TRDC respectively. The β chain constant domain is composed of one of two different β constant domains, known as TRBC1 and TRBC2 (IMGT nomenclature). There are four amino acid changes between these β constant domains, three of which are within the domains used to produce the single-chain TCRs displayed on phage particles of the present invention.

These changes are all within exon 1 of TRBC1 and TRBC2: $N_4K_5 \rightarrow K_4N_5$ and $F_{37} \rightarrow Y$

(IMGT numbering, differences TRBC1 \rightarrow TRBC2), the final amino acid change

between the two TCR β chain constant regions being in exon 3 of TRBC1 and TRBC2: V₁->E. The constant γ domain is composed of one of either TRGC1, TRGC2(2x) or TRGC2(3x). The two TRGC2 constant domains differ only in the number of copies of the amino acids encoded by exon 2 of this gene that are present.

5

The extent of each of the TCR extracellular domains is somewhat variable. However, a person skilled in the art can readily determine the position of the domain boundaries using a reference such as The T Cell Receptor Facts Book, Lefranc & Lefranc, Publ. Academic Press 2001.

10

Recombinant TCRs

The production of recombinant TCRs is beneficial as these provide soluble TCR analogues suitable for the following purposes:

15

- Studying the TCR / ligand interactions (e.g. pMHC for $\alpha\beta$ TCRs)
- Screening for inhibitors of TCR-associated interactions
- Providing the basis for potential therapeutics

20

A number of constructs have been devised to date for the production of recombinant TCRs. These constructs fall into two broad classes, single-chain TCRs and dimeric TCRs, the literature relevant to these constructs is summarised below.

Single-chain TCRs (scTCRs) are artificial constructs consisting of a single amino acid strand, which like native heterodimeric TCRs bind to MHC-peptide complexes.

25

Unfortunately, attempts to produce functional alpha/beta analogue scTCRs by simply linking the alpha and beta chains such that both are expressed in a single open reading frame have been unsuccessful, presumably because of the natural instability of the alpha-beta soluble domain pairing.

30

Accordingly, special techniques using various truncations of either or both of the alpha and beta chains have been necessary for the production of scTCRs. These formats appear to be applicable only to a very limited range of scTCR sequences. Soo Hoo *et*

al (1992) PNAS. **89** (10): 4759-63 report the expression of a mouse TCR in single chain format from the 2C T cell clone using a truncated beta and alpha chain linked with a 25 amino acid linker and bacterial periplasmic expression (see also Schodin *et al* (1996) Mol. Immunol. **33** (9): 819-29). This design also forms the basis of the m6
 5 single-chain TCR reported by Holler *et al* (2000) PNAS. **97** (10): 5387-92 which is derived from the 2C scTCR and binds to the same H2-Ld-restricted alloepitope. Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 and US 6,423,538 report using a murine single-chain 2C TCR constructs in yeast display experiments, which produced mutated TCRs with, enhanced thermal stability and solubility. This
 10 report also demonstrated the ability of these displayed 2C TCRs to selectively bind cells expressing their cognate pMHC. Khandekar *et al* (1997) J. Biol. Chem. **272** (51): 32190-7 report a similar design for the murine D10 TCR, although this scTCR was fused to MBP and expressed in bacterial cytoplasm (see also Hare *et al* (1999) Nat. Struct. Biol. **6** (6): 574-81). Hilyard *et al* (1994) PNAS. **91** (19): 9057-61 report a
 15 human scTCR specific for influenza matrix protein-HLA-A2, using a V α -linker-V β design and expressed in bacterial periplasm.

Chung *et al* (1994) PNAS. **91** (26) 12654-8 report the production of a human scTCR using a V α -linker-V β -C β design and expression on the surface of a mammalian cell
 20 line. This report does not include any reference to peptide-HLA specific binding of the scTCR. Plaksin *et al* (1997) J. Immunol. **158** (5): 2218-27 report a similar V α -linker-V β -C β design for producing a murine scTCR specific for an HIV gp120-H-2D^d epitope. This scTCR is expressed as bacterial inclusion bodies and refolded *in vitro*.

25 A number of papers describe the production of TCR heterodimers which include the native disulphide bridge which connects the respective subunits (Garboczi, *et al.*, (1996), Nature **384**(6605): 134-41; Garboczi, *et al.*, (1996), J Immunol **157**(12): 5403-10; Chang *et al.*, (1994), PNAS USA **91**: 11408-11412; Davodeau *et al.*, (1993), J. Biol. Chem. **268**(21): 15455-15460; Golden *et al.*, (1997), J. Imm. Meth. **206**: 163-
 30 169; US Patent No. 6080840). However, although such TCRs can be recognised by

TCR-specific antibodies, none were shown to recognise its native ligand at anything other than relatively high concentrations and/or were not stable.

In WO 99/60120, a soluble TCR is described which is correctly folded so that it is capable of recognising its native ligand, is stable over a period of time, and can be produced in reasonable quantities. This TCR comprises a TCR α or γ chain extracellular domain dimerised to a TCR β or δ chain extracellular domain respectively, by means of a pair of C-terminal dimerisation peptides, such as leucine zippers. This strategy of producing TCRs is generally applicable to all TCRs.

Reiter *et al*, Immunity, 1995, 2:281-287, details the construction of a soluble molecule comprising disulphide-stabilised TCR α and β variable domains, one of which is linked to a truncated form of Pseudomonas exotoxin (PE38). One of the stated reasons for producing this molecule was to overcome the inherent instability of single-chain TCRs. The position of the novel disulphide bond in the TCR variable domains was identified via homology with the variable domains of antibodies, into which these have previously been introduced (for example see Brinkmann, *et al.* (1993), Proc. Natl. Acad. Sci. USA 90: 7538-7542, and Reiter, *et al.* (1994) Biochemistry 33: 5451-5459). However, as there is no such homology between antibody and TCR constant domains, such a technique could not be employed to identify appropriate sites for new inter-chain disulphide bonds between TCR constant domains.

As mentioned above Shusta *et al* (2000) Nature Biotechnology 18: 754-759 report using single-chain 2 C TCR constructs in yeast display experiments. The principle of displaying scTCRs on phage particles has previously been discussed. For example, WO 99/19129 details the production of scTCRs, and summarise a potential method for the production of phage particles displaying scTCRs of the $V\alpha$ -Linker- $V\beta$ C β format. However, this application contains no exemplification demonstrating the production of said phage particles displaying TCR. The application does however refer to a co-pending application:

“The construction of DNA vectors including a DNA segment encoding a sc-TCR molecules fused to a bacteriophage coat protein (gene II or gene VIII) have been described in said pending U.S. application No. 08/813,781.”

5 Furthermore, this application relies on the ability of anti-TCR antibodies or super-antigen MHC complexes to recognise the soluble, non-phage displayed, scTCRs produced to verify their correct conformation. Therefore, true peptide-MHC binding specificity of the scTCRs, in any format, is not conclusively demonstrated.

10 *Screening Use*

A number of important cellular interactions and cell responses, including the TCR-mediated immune synapse, are controlled by contacts made between cell surface receptors and ligands presented on the surfaces of other cells. These types of specific molecular contacts are of crucial importance to the correct biochemical regulation in the human body and are therefore being studied intensely. In many cases, the
15 objective of such studies is to devise a means of modulating cellular responses in order to prevent or combat disease.

Therefore, methods with which to identify compounds that bind with some degree of
20 specificity to human receptor or ligand molecules are important as leads for the discovery and development of new disease therapeutics. In particular, compounds that interfere with certain receptor-ligand interactions have immediate potential as therapeutic agents or carriers.

25 Advances in combinatorial chemistry, enabling relatively easy and cost-efficient production of very large compound libraries have increased the scope for compound testing enormously. Now the limitations of screening programmes most often reside in the nature of the assays that can be employed, the production of suitable receptor and ligand molecules and how well these assays can be adapted to high throughput
30 screening methods.

Display Methods

It is often desirable to present a given peptide or polypeptide on the surface of a proteinaceous particle. Such particles may serve as purification aids for the peptide or polypeptide (since the particles carrying the peptide or polypeptide may be separated from unwanted contaminants by sedimentation or other methods). They may also serve as particulate vaccines, the immune response to the surface displayed peptide or polypeptide being stimulated by the particulate presentation. Protein p24 of the yeast retrotransposon, and the hepatitis B surface coat protein are examples of proteins which self assemble into particles. Fusion of the peptide or polypeptide of interest to these particle-forming proteins is a recognised way of presenting the peptide or polypeptide on the surface of the resultant particles.

However, particle display methods have primarily been used to identify proteins with desirable properties such as enhanced expression yields, binding and/or stability characteristics. These methods involve creating a diverse pool or 'library' of proteins or polypeptides expressed on the surface of proteinaceous particles. These particles have two key features, firstly each particle presents a single variant protein or polypeptide, and secondly the genetic material encoding the expressed protein or polypeptide is associated with that of the particle. This library is then subjected to one or more rounds of selection. For example, this may consist of contacting a ligand with a particle-display library of mutated receptors and identifying which mutated receptors bind the ligand with the highest affinity. Once the selection process has been completed the receptor or receptors with the desired properties can be isolated, and their genetic material can be amplified in order to allow the receptors to be sequenced.

These display methods fall into two broad categories, *in-vitro* and *in-vivo* display.

All *in-vivo* display methods rely on a step in which the library, usually encoded in or with the genetic nucleic acid of a replicable particle such as a plasmid or phage replicon is transformed into cells to allow expression of the proteins or polypeptides. (Plückthun (2001) Adv Protein Chem 55 367-403). There are a number of

replicon/host systems that have proved suitable for *in-vivo* display of protein or polypeptides. These include the following

Phage / bacterial cells
5 plasmid / CHO cells

Vectors based on the yeast 2 μ m plasmid / yeast cells
bacculovirus / insect cells
10 plasmid / bacterial cells

15 In-vivo display methods include cell-surface display methods in which a plasmid is introduced into the host cell encoding a fusion protein consisting of the protein or polypeptide of interest fused to a cell surface protein or polypeptide. The expression of this fusion protein leads to the protein or polypeptide of interest being displayed on the surface of the cell. The cells displaying these proteins or polypeptides of interest can then be subjected to a selection process such as FACS and the plasmids obtained from the selected cell or cells can be isolated and sequenced. Cell surface display systems have been devised for mammalian cells (Higuchi (1997) J Immunol. Methods **202** 193-204), yeast cells (Shusta (1999) J Mol Biol **292** 949-956) and
20 bacterial cells (Sameulson (2002) J. Biotechnol **96** (2) 129-154).

Numerous reviews of the various in-vivo display techniques have been published. For example, (Hudson (2002) Expert Opin Biol Ther (2001) **1** (5) 845-55) and (Schmitz (2000) **21** (Supp A) S106-S112).

25 *In-vitro* display methods are based on the use of ribosomes to translate libraries of mRNA into a diverse array of protein or polypeptide variants. The linkage between the proteins or polypeptides formed and the mRNA encoding these molecules is maintained by one of two methods. Conventional ribosome display utilises mRNA
30 sequences that encode a short (typically 40-100-amino-acid-) linker sequence and the protein or polypeptide to be displayed. The linker sequence allow the displayed

protein or polypeptide sufficient space to re-fold without being sterically hindered by the ribosome. The mRNA sequence lacks a 'stop' codon, this ensures that the expressed protein or polypeptide and the RNA remain attached to the ribosome particle. The related mRNA display method is based on the preparation of mRNA sequences encoding the protein or polypeptide of interest and DNA linkers carrying a puromycin moiety. As soon as the ribosome reaches the mRNA/DNA junction translation is stalled and the puromycin forms a covalent linkage to the ribosome. For a recent review of these two related *in-vitro* display methods see (Amstutz (2001) Curr Opin Biotechnol 12 400-405).

Particularly preferred is the phage display technique which is based on the ability of bacteriophage particles to express a heterologous peptide or polypeptide fused to their surface proteins. (Smith (1985) Science 217 1315-1317). The procedure is quite general, and well understood in the art for the display of polypeptide monomers. However, in the case of polypeptides that in their native form associate as dimers, only the phage display of antibodies appears to have been thoroughly investigated.

For monomeric polypeptide display there are two main procedures:

Firstly (Method A) by inserting into a vector (phagemid) DNA encoding the heterologous peptide or polypeptide fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by transfecting bacterial cells with the phagemid, and then infecting the transformed cells with a 'helper phage'. The helper phage acts as a source of the phage proteins not encoded by the phagemid required to produce a functional phage particle.

Secondly (Method B), by inserting DNA encoding the heterologous peptide or polypeptide into a complete phage genome fused to the DNA encoding a bacteriophage coat protein. The expression of phage particles displaying the heterologous peptide or polypeptide is then carried out by infecting bacterial cells with the phage genome. This method has the advantage of the first method of being a

'single-step' process. However, the size of the heterologous DNA sequence that can be successfully packaged into the resulting phage particles is reduced. M13, T7 and Lambda are examples of suitable phages for this method.

5 A variation on (Method B) the involves adding a DNA sequence encoding a nucleotide binding domain to the DNA in the phage genome encoding the heterologous peptide to be displayed, and further adding the corresponding nucleotide binding site to the phage genome. This causes the heterologous peptide to become directly attached to the phage genome. This peptide/genome complex is then packaged
10 into a phage particle which displays the heterologous peptide. This method is fully described in WO 99/11785.

The phage particles can then be recovered and used to study the binding characteristics of the heterologous peptide or polypeptide. Once isolated, phagemid or phage DNA
15 can be recovered from the peptide- or polypeptide-displaying phage particle, and this DNA can be replicated via PCR. The PCR product can be used to sequence the heterologous peptide or polypeptide displayed by a given phage particle.

The phage display of single-chain antibodies and fragments thereof, has become a
20 routine means of studying the binding characteristics of these polypeptides. There are numerous books available that review phage display techniques and the biology of the bacteriophage. (See, for example, Phage Display – A Laboratory Manual, Barbas *et al.*, (2001) Cold Spring Harbour Laboratory Press).

25 A third phage display method (Method C) relies on the fact that heterologous polypeptides having a cysteine residue at a desired location can be expressed in a soluble form by a phagemid or phage genome, and caused to associate with a modified phage surface protein also having a cysteine residue at a surface exposed position, via the formation of a disulphide linkage between the two cysteines. WO 01/ 05950 details
30 the use of this alternative linkage method for the expression of single-chain antibody-derived peptides.

Brief Description of the Invention

Native TCR's are heterodimers which have lengthy transmembrane domains which are essential to maintain their stability as functional dimers. As discussed above, TCRs are useful for research and therapeutic purposes in their soluble forms so display of the insoluble native form has little utility. On the other hand, soluble stable forms of TCRs have proved difficult to design, and since most display methods appear to have been described only for monomeric peptides and polypeptides, display methods suitable for soluble dimeric TCRs have not been investigated. Furthermore, since the functionality of the displayed TCR depends on proper association of the variable regions of the TCR dimer, successful display of a functional dimeric TCR is not trivial.

WO 99/18129 contains the statement : "DNA constructs encoding the sc-TCR fusion proteins can be used to make a bacteriophage display library in accordance with methods described in pending U.S. application Serial No. 08/813.781 filed on March 7, 1997, the disclosure of which is incorporated herein by reference.", but no actual description of such display is included in this application. However, The inventors of this application published a paper (Weidanz (1998) J Immunol Methods 221 59-76) that demonstrates the display of two murine scTCRs on phage particles.

WO 01/62908 discloses methods for the phage display of scTCRs and scTCR/ Ig fusion proteins. However, the functionality (specific pMHC binding) of the constructs disclosed was not assessed.

Finally, a retrovirus-mediated method for the display of diverse TCR libraries on the surface of immature T cells has been demonstrated for a murine TCR. The library of mutated TCRs displayed of the surface of the immature T cells was screened by flow cytometry using pMHC tetramers, and this lead to the identification TCR variants that were either specific for the cognate pMHC, or a variant thereof. (Helmut *et al.*, (2000) PNAS 97 (26) 14578-14583)

This invention is based in part on the finding that single chain and dimeric TCRs can be expressed as surface fusions to proteinaceous particles, and makes available proteinaceous particles displaying alpha/beta-analogue and gamma/delta-analogue scTCR and dTCR constructs. The proteinaceous particles on which the TCRs are displayed include self-aggregating particle-forming proteins, phage, virus-derived and ribosome particles. Such proteinaceous particle-displayed TCRs are useful for purification and screening purposes, particularly as a diverse library of particle displayed TCRs for biopanning to identify TCRs with desirable characteristics such as strong affinity for the target MHC-peptide complex. In the latter connection, particle-displayed scTCRs may be useful for identification of the desired TCR, but that information may be better applied to the construction of analogous dimeric TCRs for ultimate use in therapy.

Detailed Description of the Invention

The present invention provides a proteinaceous particle displaying on its surface a T-cell receptor(TCR), characterised in that

(i) the proteinaceous particle is a ribosome and the TCR is a single chain TCR (scTCR) polypeptide, or

(ii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a human scTCR or a human dimeric T-cell receptor (dTCR) polypeptide pair, or

(iii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a non-human dTCR polypeptide pair, or

(iv) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a scTCR polypeptide comprising TCR

amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the

latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain, and a disulfide bond which has no equivalent in native T cell receptors links residues of the constant region sequences.

In a preferred embodiment, the invention provides a proteinaceous particle displaying on its surface a dimeric T-cell receptor (dTCR) polypeptide pair, or a single chain T-cell receptor (scTCR) polypeptide wherein

the dTCR polypeptide pair is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains, and the scTCR is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain;

the variable region sequences of the dTCR polypeptide pair or scTCR polypeptide are mutually orientated substantially as in native TCRs; and

in the case of the scTCR polypeptide a disulfide bond which has no equivalent in native T cell receptors links residues of the polypeptide.

In the case of $\alpha\beta$ scTCRs or dTCRs displayed according to the invention, the requirement that the variable region sequences of the α and β segments are mutually orientated substantially as in native $\alpha\beta$ T cell receptors is tested by confirming that the molecule binds to the relevant TCR ligand (pMHC complex, CD1-antigen complex, superantigen or superantigen/pMHC complex) - if it binds, then the requirement is met. Interactions with pMHC complexes can be measured using a BIAcore 3000™ or

BIAcore 2000™ instrument. WO99/6120 provides detailed descriptions of the methods required to analyse TCR binding to MHC-peptide complexes. These methods are equally applicable to the study of TCR/ CD1 and TCR/superantigen interactions. In order to apply these methods to the study of TCR/CD1 interactions soluble forms of CD1 are required, the production of which are described in (Bauer (1997) Eur J Immunol 27 (6) 1366-1373). In the case of $\gamma\delta$ TCRs of the present invention the cognate ligands for these molecules are unknown therefore secondary means of verifying their conformation such as recognition by antibodies can be employed. The monoclonal antibody MCA991T (available from Serotec), specific for the δ chain variable region, is an example of an antibody appropriate for this task.

The scTCRs or dTCRs of the present invention may be displayed on phage particles by, for example, the following two means:

- (i) The C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, can be directly linked by a peptide bond to a surface exposed residue of the proteinaceous particle. For example, the said surface exposed residue is preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII; and
- (ii) The C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, is linked by a disulfide bond to a surface exposed cysteine residue of the proteinaceous particle via an introduced cysteine residue. For example, the said surface exposed residue is again preferably at the N-terminus of the gene product of bacteriophage gene III or gene VIII.

Method (i) above is preferred. In the case of a scTCR, nucleic acid encoding the TCR may be fused to nucleic acid encoding the particle forming protein or a surface protein of the replicable particle such as a phage. Alternatively, nucleic acid representing mRNA but without a stop codon, or fused to puromycin RNA may be translated by a ribosome such that the TCR remains fused to the ribosome particle. In the case of a

dTCR, nucleic acid encoding one chain of the TCR may be fused to nucleic acid encoding the particle forming protein or a surface protein of the replicable particle such as a phage, and the second chain of the TCR polypeptide pair may be allowed to associate with the resultant expressed particle displaying the first chain. Proper functional association of the two chains is assisted by the presence of cysteines in the constant region of the two chains which are capable of forming an interchain disulfide bond, as more fully discussed below.

The displayed scTCR

The displayed scTCR polypeptide may be, for example, one which has a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence,

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant region extracellular sequence,

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment, or vice versa, and

a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native $\alpha\beta$ or $\gamma\delta$ T cell receptors,

the length of the linker sequence and the position of the disulfide bond being such that the variable region sequences of the first and second segments are mutually orientated substantially as in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

The displayed dTCR

The dTCR which is displayed on the proteinaceous particle may be one which is constituted by

5 a first polypeptide wherein a sequence corresponding to a TCR α or δ chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant region extracellular sequence, and

10 a second polypeptide wherein a sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus a sequence corresponding to a TCR β chain constant region extracellular sequence,

the first and second polypeptides being linked by a disulfide bond which has no equivalent in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

15 *dTCR Polypeptide Pair and scTCR Polypeptide*

The constant region extracellular sequences present in the scTCRs or dTCRs preferably correspond to those of a human TCR, as do the variable region sequences. However, the correspondence between such sequences need not be 1:1 on an amino acid level. N- or C-truncation, and/or amino acid deletion and/or substitution relative to the corresponding human TCR sequences is acceptable, provided the overall result is mutual orientation of the α and β variable region sequences, or γ and δ variable region sequences is as in native $\alpha\beta$, or $\gamma\delta$ T cell receptors respectively. In particular, because the constant region extracellular sequences present in the first and second segments are not directly involved in contacts with the ligand to which the scTCR or dTCR binds, they may be shorter than, or may contain substitutions or deletions relative to, extracellular constant domain sequences of native TCRs.

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The constant region extracellular sequence present in one of the dTCR polypeptide pair, or in the first segment of a scTCR polypeptide may include a sequence

30 corresponding to the extracellular constant Ig domain of a TCR α chain, and/or the

constant region extracellular sequence present in the other member of the pair or second segment may include a sequence corresponding to the extracellular constant Ig domain of a TCR β chain.

5 In one embodiment of the invention, one member of the polypeptide pair or the first segment of the scTCR polypeptide corresponds to substantially all the variable region of a TCR α chain fused to the N terminus of substantially all the extracellular domain of the constant region of an TCR α chain; and/or the other member of the pair or second segment corresponds to substantially all the variable region of a TCR β chain
10 fused to the N terminus of substantially all the extracellular domain of the constant region of a TCR β chain.

In another embodiment, the constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide
15 correspond to the constant regions of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native inter-chain disulfide bond of the TCR are excluded. Alternatively those cysteine residues may be substituted by another amino acid residue such as serine or alanine, so that the native disulfide bond is deleted. In addition, the native TCR β chain contains an unpaired
20 cysteine residue and that residue may be deleted from, or replaced by a non-cysteine residue in, the β sequence of the scTCR of the invention.

In one particular embodiment of the invention, the TCR α and β chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the
25 scTCR polypeptide may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus the α and β chain variable region sequences present in dTCR
30 polypeptide pair or first and second segments of the scTCR polypeptide may

correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant region extracellular sequences can be used as a framework onto which heterologous α and β variable domains can be fused.

5

In another embodiment of the invention, the TCR δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may together correspond to the functional variable domain of a first TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide respectively, may correspond to those of a second TCR, the first and second TCRs being from the same species. Thus the δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second human TCR. For example, A6 Tax sTCR constant region extracellular sequences can be used as a framework onto which heterologous γ and δ variable domains can be fused.

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In one particular embodiment of the invention, the TCR α and β , or δ and γ chain variable region sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may together correspond to the functional variable domain of a first human TCR, and the TCR α and β chain constant region extracellular sequences present in the dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a second non-human TCR. Thus the α and β , or δ and γ chain variable region sequences present dTCR polypeptide pair or first and second segments of the scTCR polypeptide may correspond to those of a first human TCR, and the α and β chain constant region extracellular sequences may correspond to those of a second non-human TCR. For example, murine TCR constant

region extracellular sequences can be used as a framework onto which heterologous human α and β TCR variable domains can be fused.

Linker in the scTCR Polypeptide

5 For scTCR-displaying proteinaceous particles of the present invention, a linker sequence links the first and second TCR segments, to form a single polypeptide strand. The linker sequence may, for example, have the formula -P-AA-P- wherein P is proline and AA represents an amino acid sequence wherein the amino acids are glycine and serine.

10

For the scTCR displayed by proteinaceous particles of the present invention to bind to a ligand, MHC-peptide complex in the case of $\alpha\beta$ TCRs, the first and second segments must be paired so that the variable region sequences thereof are orientated for such binding. Hence the linker should have sufficient length to span the distance between the C terminus of the first segment and the N terminus of the second segment, or vice versa. On the other hand excessive linker length should preferably be avoided, in case the end of the linker at the N-terminal variable region sequence blocks or reduces bonding of the scTCR to the target ligand.

15

20 For example, in the case where the constant region extracellular sequences present in the first and second segments correspond to the constant regions of the α and β chains of a native TCR truncated at their C termini such that the cysteine residues which form the native interchain disulfide bond of the TCR are excluded, and the linker sequence links the C terminus of the first segment to the N terminus of the second segment, the linker may consist of from 26 to 41, for example 29, 30, 31 or 32 amino acids, and a particular linker has the formula -PGGG-(SGGGG)₅-P- wherein P is proline, G is glycine and S is serine.

25

Inter-chain Disulfide bond

30 A principle characterising feature of the scTCRs displayed by proteinaceous particles of the present invention, and preferably a feature of the displayed dTCRs, is the a

disulfide bond between the constant region extracellular sequences of the dTCR polypeptide pair or first and second segments of the scTCR polypeptide. That bond may correspond to the native inter-chain disulfide bond present in native dimeric $\alpha\beta$ TCRs, or may have no counterpart in native TCRs, being between cysteines specifically incorporated into the constant region extracellular sequences of dTCR polypeptide pair or first and second segments of the scTCR polypeptide. In some cases, both a native and a non-native disulfide bond may be desirable.

The position of the disulfide bond is subject to the requirement that the variable region sequences of dTCR polypeptide pair or first and second segments of the scTCR polypeptide are mutually orientated substantially as in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

The disulfide bond may be formed by mutating non-cysteine residues on the first and second segments to cysteine, and causing the bond to be formed between the mutated residues. Residues whose respective β carbons are approximately 6 Å (0.6 nm) or less, and preferably in the range 3.5 Å (0.35 nm) to 5.9 Å (0.59 nm) apart in the native TCR are preferred, such that a disulfide bond can be formed between cysteine residues introduced in place of the native residues. It is preferred if the disulfide bond is between residues in the constant immunoglobulin region, although it could be between residues of the membrane proximal region. Preferred sites where cysteines can be introduced to form the disulfide bond are the following residues in exon 1 of TRAC*01 for the TCR α chain and TRBC1*01 or TRBC2*01 for the TCR β chain:

TCR α chain	TCR β chain	Native β carbon separation (nm)
Thr 48	Ser 57	0.473
Thr 45	Ser 77	0.533
Tyr 10	Ser 17	0.359
Thr 45	Asp 59	0.560
Ser 15	Glu 15	0.59

Now that the residues in human TCRs which can be mutated into cysteine residues to form a new interchain disulfide bond in dTCRs or scTCRs displayed according to the invention have been identified, those of skill in the art will be able to mutate TCRs of other species in the same way to produce a dTCR or scTCR of that species for phage display. In humans, the skilled person merely needs to look for the following motifs in the respective TCR chains to identify the residue to be mutated (the shaded residue is the residue for mutation to a cysteine).

10	α Chain Thr 48:	DSDVYITDKTVLDMRSMDFK (amino acids 39-58 of exon 1 of the TRAC*01 gene)
	α Chain Thr 45:	QSKDSDVYITDKTVLDMRSM(amino acids 36-55 of exon 1 of the TRAC*01 gene)
15	α Chain Tyr 10:	DIQNPDPAVYQLRDSKSSDK(amino acids 1-20 of exon 1 of the TRAC*01 gene)
20	α Chain Ser 15:	DPAVYQLRDSKSSDKSVCLF(amino acids 6-25 of exon 1 of the TRAC*01 gene)
	β Chain Ser 57:	NGKEVHSGVSTDPQPLKEQP(amino acids 48- 67 of exon 1 of the TRBC1*01 & TRBC2*01 genes)
25	β Chain Ser 77:	ALNDSRYALSSRLRVSAATFW(amino acids 68- 87 of exon 1 of the TRBC1*01 & TRBC2*01 genes)
	β Chain Ser 17:	PPEVAVFEPSEAEISHTQKA(amino acids 8- 27 of exon 1 of the TRBC1*01 & TRBC2*01 genes)
30	β Chain Asp 59:	KEVHSGVSTDPQPLKEQPAL(amino acids 50- 69 of exon 1 of the TRBC1*01 & TRBC2*01 genes gene)

β Chain Glu 15: VFPPEVAVFEPSEAEISHTQ(amino acids 6- 25 of exon 1 of the TRBC1*01 & TRBC2*01 genes)

5 In other species, the TCR chains may not have a region which has 100% identity to the above motifs. However, those of skill in the art will be able to use the above motifs to identify the equivalent part of the TCR α or β chain and hence the residue to be mutated to cysteine. Alignment techniques may be used in this respect. For example, ClustalW, available on the European Bioinformatics Institute website
10 (<http://www.ebi.ac.uk/index.html>) can be used to compare the motifs above to a particular TCR chain sequence in order to locate the relevant part of the TCR sequence for mutation.

The present invention includes within its scope proteinaceous particle-displayed $\alpha\beta$
15 and $\gamma\delta$ -analogue scTCRs, as well as those of other mammals, including, but not limited to, mouse, rat, pig, goat and sheep. As mentioned above, those of skill in the art will be able to determine sites equivalent to the above-described human sites at which cysteine residues can be introduced to form an inter-chain disulfide bond. For example, the following shows the amino acid sequences of the mouse C α and C β
20 soluble domains, together with motifs showing the murine residues equivalent to the human residues mentioned above that can be mutated to cysteines to form a TCR interchain disulfide bond (where the relevant residues are shaded):

Mouse C α soluble domain:

25 PYIQNPEPAVYQLKDPRSQDSTLCLFTDFDSQINVPKTMESGTFITDKTVLDMK
AMDSKSNGAIAWSNQTSFTCQDIFKETNATYPSSDVP

Mouse C β soluble domain:

EDLRNVTPPKVSLFEP SKAEIANKQKATLVCLARGFFPDHVELSWVNGREV
30 HSGVSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDK
WPEGSPKPV TQNISAEAWGRAD

Murine equivalent of human α Chain Thr 48: ESGTFITDKTVLDMKAMDSK

Murine equivalent of human α Chain Thr 45: KTMESGTFITDKTVLDMKAM

5

Murine equivalent of human α Chain Tyr 10: YIQNPEPAVYQLKDPRSQDS

Murine equivalent of human α Chain Ser 15: AVYQLKDPRSQDSTLCLFTD

10

Murine equivalent of human β Chain Ser 57: NGREVVHSGVSTDPQAYKESN

Murine equivalent of human β Chain Ser 77: KESNYSYCLSSRLRVSATFW

Murine equivalent of human β Chain Ser 17: PPKVSLFEPSKAEIANKQKA

15

Murine equivalent of human β Chain Asp 59: REVHSGVSTDPQAYKESNYS

Murine equivalent of human β Chain Glu 15: VTPPKVSLFEPSKAEIANKQ

20

As discussed above, the A6 Tax sTCR extracellular constant regions can be used as framework onto which heterologous variable domains can be fused. It is preferred that the heterologous variable region sequences are linked to the constant region sequences at any point between the disulfide bond and the N termini of the constant region sequences. In the case of the A6 Tax TCR α and β constant region sequences, the disulfide bond may be formed between cysteine residues introduced at amino acid residues 158 and 172 respectively. Therefore it is preferred if the heterologous α and β chain variable region sequence attachment points are between residues 159 or 173 and the N terminus of the α or β constant region sequences respectively.

25

TCR Display.

The preferred *in-vivo* TCR display method for biopanning to identify TCRs having desirable properties such as strong affinity for a target peptide-MHC complex is phage display.

Firstly, a DNA library is constructed that encodes a diverse array of mutated scTCRs or dTCRs. This library is constructed by using DNA encoding a native TCR as the template for amplification. There are a number of suitable methods, known to those skilled in the art, for the introduction of the desired mutations into the TCR DNA, and hence the finally expressed TCR protein. For example error-prone PCR (EP-TCR), DNA shuffling techniques, and the use of bacterial mutator strains such as XL-1-Red are convenient means of introducing mutations into the TCR sequences. It is particularly preferred if these mutations are introduced into defined regions of the TCRs. For example, mutations in the variable region, particularly the complementarity-determining regions (CDRs) are likely to be the most appropriate sites for the introduction of mutations leading to the production of a diverse library of TCRs for the production of TCRs with enhanced ligand-binding properties. EP-PCR is an example of a method by which such 'region-specific' mutations can be introduced into the TCRs. EP-PCR primers are used that are complementary to DNA sequences bordering the region to be mutated to amplify multiple copies of this region of the TCR DNA that contain a controllable level of random mutations. These DNA sequences encoding mutated regions are inserted into the DNA sequences, which encode the non-mutagenised sections of the TCR, by ligation or overlapping PCR. The DNA encoding the TCR with mutated region can then be ligated onto DNA encoding a heterologous polypeptide in order to produce a fusion protein suitable for display. In the case of phage-display the expression vector utilised is either a phagemid or a phage genome vector in which the TCR DNA can be ligated to DNA encoding the gIII or gVIII surface protein. In the case of a scTCR such ligation is performed as for phage display of any monomeric peptide or polypeptide. In the case of dTCRs, only one of the TCR chains is ligated as aforesaid. The other chain is

encoded in nucleic acid for co-expression with the phagemid and helper phage nucleic acid, so that the expressed second chain finds and associates with the expressed phage with surface displayed first chain. In both cases, as discussed in more detail above, properly positioned cysteines in the constant regions are helpful in causing the variable regions of the TCR to adopt their functional positions, through the formation of a disulfide bond by those cysteines.

For expression, the expression vectors comprising the TCR DNA library are then contacted with host cells capable of causing the expression of the encoded genetic material under conditions suitable to allow the transformation of said cells. Such expression vectors and host cells harbouring them from additional aspects of the current invention.

The transformed cells are then incubated to allow the expression of the TCR-displaying proteinaceous particles. These particles can then be used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The DNA encoding these TCRs can then be amplified by PCR and the sequence determined.

It is known that high expression levels of an exogenous polypeptide may be toxic to the host cell. In such cases, either a host strain which is more tolerant of the exogenous polypeptide must be found, or the expression levels in the host cell must be limited to a level which is tolerated. For example (Beekwilder *et al.*, (1999) Gene 228 (1-2) 23-31) report that only mutated forms of a potato protease inhibitor (PI2) which contained deletions or amber stop codons would be successfully selected from a phage display library. In the present case, an observation in the course of the work reported in the Examples herein suggests that it may be desirable to limit the expression levels of protein particle-displayed TCRs of the invention, at least in some strains of *E. coli*. Thus, the A6 TCR selected in Example 4 after repeated rounds of culture was shown to be derived from cells in which the phagemid had mutated relative to that introduced

at the start. The mutation had created an 'opal' stop codon in the TCR β chain. This codon is 'read-through' with low frequency by ribosomes of the *E.coli* strain utilised resulting in the insertion of a tryptophan residue at this site and a much reduced overall level of full-length β chain expression.

5

There are several strategies for limiting the expression levels of an exogenous polypeptide from a given expression vector in a host which may be suitable for the limiting the expression levels of a scTCR, or one, or both TCR chains of a dTCR . For example:

10

Use of a weak promoter sequence – The level of expression obtained for a given gene product, such as the TCR α or β chain, can be tailored by using promoter sequences of varying strengths. The lambda phage P_{RM} promoter is an example of a weak promoter.

15

Mutated ribosome binding sites (RBS's) – Mutating a single nucleic acid in the RBS associated with a gene product, such as the TCR α or β chain, can result in a reduced level of expression. For example, mutating a wild-type AGGA sequence to AGGG.

20

Mutated 'start codons' - Mutating a single nucleic acid in the start codon associated with a gene product, such as the TCR α or β chain, can also result in a reduced level of expression. For example, mutating a wild-type AUG start codon to GUG.

25

Miss-sense suppressor mutations – These are inserted within the TCR β chain coding regions. Examples include the 'opal' stop codon (UGA), this 'leaky' stop codon results in the low frequency insertion of a tryptophan amino acid and read-through of the rest of the coding sequence.

30

Metabolite-mediated modification of promoter strength – The level of expression of a gene product, such as the TCR α or β chain, under the control of certain promoters can be down-regulated by the addition of a relevant metabolite to the cells containing the

promoter. For example, glucose additions can be used to down-regulate expression of a gene product under the control of a Lac promoter.

5 Codon usage - Bacterial cells and, for example, mammalian cells have different 'preferences' relating to the codons they use to encode certain amino acids. For example, bacterial cells most commonly use the CGU codon to encode arginine whereas eucaryotic cells most commonly use AGA. It is possible to reduce the level of expression of a gene product, such as the TCR α or β chain, by utilising DNA sequences that contain a number of codons less preferred by the expression system
10 being utilised.

Details relating to the above means of down-regulating gene product expression can be found in (Glass (1982) Gene Function – *E.coli* and its heritable elements, Croom Helm) and (Rezinoff (1980) The Operon 2nd Edition, Cold Spring Harbor Laboratory).

15 It is also known that supplying bacterial cultures with a relatively high concentration of a sugar such as sucrose can increase periplasmic expression levels of soluble proteins. (See for example (Sawyer *et al.*, (1994) Protein Engineering 7 (11) 1401-1406))

20 As mentioned above, for scTCR phage display, the scTCR polypeptide is expressed according to any of the three general prior art techniques discussed earlier as Methods A, B, and C. For the scTCRs displayed by phage particles of the present invention to bind to a ligand, MHC-peptide complex in the case of $\alpha\beta$ TCRs, the first and second
25 segments must be paired so that the variable region sequences thereof are orientated for such binding. This correct pairing is assisted by the introduction of a disulfide bond in the extracellular constant region of the scTCR. Without wanting to be limited by theory, the novel disulfide bond is believed to provide extra stability to the scTCR during the folding process and thereby facilitating correct pairing of the first and
30 second segments.

Also as mentioned above, for dTCR phage display, one of the dTCR polypeptide pair is expressed according to any of the three general prior art techniques discussed earlier as Methods A, B, and C as if it were eventually to be displayed as a monomeric polypeptide on the phage, and the other of the dTCR polypeptide pair is co-expressed in the same host cell. As the phage particle self assembles, the two polypeptides self associate for display as a dimer on the phage. Again, in the preferred embodiment of this aspect of the invention, correct folding during association of the polypeptide pair is assisted by a disulfide bond between the constant sequences, as discussed above. Further details of a procedure for phage display of a dTCR having an interchain disulfide bond appear in the Examples herein.

As an alternative, the phage displaying the first chain of the TCR may be expressed first, and the second chain polypeptide may be contacted with the expressed phage in a subsequent step, for association as a functional TCR on the phage surface.

The preferred *in-vitro* TCR display method for biopanning to identify TCRs having desirable properties such as strong affinity for a target peptide-MHC complex is ribosomal display of scTCRs.

Firstly, a DNA library is constructed that encodes a diverse array of mutated TCRs using the techniques discussed above.

The DNA library is then contacted with RNA polymerase in order to produce a complementary mRNA library. Optionally, for mRNA display techniques the mRNA sequences can then be ligated to a DNA sequence comprising a puromycin binding site. These genetic constructs are then contacted with ribosomes *in-vitro* under conditions allowing the translation of the scTCR polypeptide. These scTCR-displaying ribosomes can then be used for screening or in assays to identify TCR variants with specific enhanced characteristics. Any particles that possess the enhanced characteristics under investigation can then be isolated. The mRNA encoding these TCRs can then be converted to the complementary DNA sequences using reverse transcriptase. This DNA can then be amplified by PCR and the sequence determined.

Additional Aspects

5 A proteinaceous particle displaying a scTCR or dTCR (which preferably is constituted by constant and variable sequences corresponding to human sequences) of the present invention may be provided in substantially pure form, or as a purified or isolated preparation. For example, it may be provided in a form which is substantially free of other proteins.

10 A phage particle displaying a plurality of scTCRs or dTCRs of the present invention may be provided in a multivalent complex. Thus, the present invention provides, in one aspect, a multivalent T cell receptor (TCR) complex, which comprises a phage particle displaying a plurality of scTCRs or dTCRs as described herein. Each of the plurality of said scTCRs or dTCRs is preferably identical.

15

In a further aspect, the invention provides a method for detecting TCR ligand complexes, which comprises:

- a. providing a TCR-displaying proteinaceous particle of the current invention
- 20 b. contacting the TCR-displaying phage with the putative ligand complexes; and detecting binding of the TCR-displaying proteinaceous particle to the putative ligand complexes.

25 TCR ligands suitable for identification by the above method include, but are not limited to, peptide-MHC complexes.

Isolation of TCR variants with enhanced characteristics

30 A further aspect of the invention is a method for the isolation of TCRs with a specific enhanced characteristic, said method comprising subjecting a diverse library of TCRs displayed on proteinaceous particles to an assay which measures said characteristic and thereby identifying those proteinaceous particles which display a TCR with the

desired enhancement and isolating these proteinaceous particles. The DNA sequences encoding the variant TCRs can then be obtained and amplified by PCR to allow the sequences to be determined. The characteristics that can be enhanced include, but are not limited to, ligand binding affinity and construct stability.

5

Screening Use

The TCR-displaying proteinaceous particles of the present invention are capable of utilisation in screening methods designed to identify modulators, including inhibitors, of the TCR-mediated cellular immune synapse.

10

As is known to those skilled in the art there are a number of assay formats that provide a suitable basis for protein-protein interaction screens of this type.

15

Amplified Luminescent Proximity Homogeneous Assay systems such as the AlphaScreen™, rely on the use of "Donor" and "Acceptor" beads that are coated with a layer of hydrogel to which receptor and ligand proteins can be attached. The interaction between these receptor and ligand molecules brings the beads into proximity. When these beads are subject to laser light a photosensitizer in the "Donor" bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a chemiluminescer in the "Acceptor" bead that further activates fluorophores contained within the same bead. The fluorophores subsequently emit light at 520-620 nm, this signals that the receptor-ligand interaction has occurred. The presence of an inhibitor of the receptor-ligand interaction causes this signal to be diminished.

20

25

Surface Plasmon Resonance (SPR) is an interfacial optical assay, in which one binding partner (normally the receptor) is immobilised on a 'chip' (the sensor surface) and the binding of the other binding partner (normally the ligand), which is soluble and is caused to flow over the chip, is detected. The binding of the ligand results in an

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increase in concentration of protein near to the chip surface which causes a change in the refractive index in that region. The surface of the chip is comprised such that the

change in refractive index may be detected by surface plasmon resonance, an optical phenomenon whereby light at a certain angle of incidence on a thin metal film produces a reflected beam of reduced intensity due to the resonant excitation of waves of oscillating surface charge density (surface plasmons). The resonance is very sensitive to changes in the refractive index on the far side of the metal film, and it is this signal which is used to detect binding between the immobilised and soluble proteins. Systems which allow convenient use of SPR detection of molecular interactions, and data analysis, are commercially available. Examples are the IasysTM machines (Fisons) and the BiacoreTM machines.

Other interfacial optical assays include total internal reflectance fluorescence (TIRF), resonant mirror (RM) and optical grating coupler sensor (GCS), and are discussed in more detail in Woodbury and Venton (*J. Chromatog. B.* **725** 113-137 (1999)).

The scintillation proximity assay (SPA) has been used to screen compound libraries for inhibitors of the low affinity interaction between CD28 and B7 (K_d probably in the region of 4 μ M (Van der Merwe *et al* *J. Exp. Med.* **185**:393-403 (1997), Jenh *et al*, *Anal Biochem* **165**(2) 287-93 (1998)). SPA is a radioactive assay making use of beta particle emission from certain radioactive isotopes which transfers energy to a scintillant immobilised on the indicator surface. The short range of the beta particles in solution ensures that scintillation only occurs when the beta particles are emitted in close proximity to the scintillant. When applied for the detection of protein-protein interactions, one interaction partner is labelled with the radioisotope, while the other is either bound to beads containing scintillant or coated on a surface together with scintillant. If the assay can be set up optimally, the radioisotope will be brought close enough to the scintillant for photon emission to be activated only when binding between the two proteins occurs.

A further aspect of the invention is a method of identifying an inhibitor of the interaction between a TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand comprising contacting the TCR-displaying proteinaceous particle with a TCR-binding ligand, in the presence of and in the absence of a test compound,

and determining whether the presence of the test compound reduces binding of the TCR-displaying proteinaceous particle to the TCR-binding ligand, such reduction being taken as identifying an inhibitor.

5 A further aspect of the invention is a method of identifying a potential inhibitor of the interaction between an TCR-displaying proteinaceous particle of the invention, and a TCR-binding ligand, for example a MHC-peptide complex, comprising contacting the TCR-displaying proteinaceous particle or TCR-binding ligand partner with a test compound and determining whether the test compound binds to the TCR-displaying
10 proteinaceous particle and/or the TCR-binding ligand, such binding being taken as identifying a potential inhibitor. This aspect of the invention may find particular utility in interfacial optical assays such as those carried out using the BIAcore™ system.

High Affinity TCRs

15 The present invention also makes available mutated TCRs specific for a given TCR ligand with higher affinity for said TCR ligand than the wild-type TCR. These high affinity TCRs are expected to be particularly useful for the diagnosis and treatment of disease.

20 Where used herein the term 'high affinity TCR' is defined as as any mutated sc or dTCR of the present invention which interacts with a specific TCR ligand and either:
has a K_d for the said TCR ligand less than that of the native TCR as measured by Surface Plasmon Resonance.

25

Or

has an off-rate (k_{off}) for the said TCR ligand less than that of the native TCR as measured by Surface Plasmon Resonance.

High affinity scTCRs or dTCRs of the present invention are preferably mutated relative to the native TCR in at least one complementarity determining region.

5 In one aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is a pMHC.

In a further aspect of the present invention the TCR ligand for which a given high affinity TCR is specific is the HLA-A2 tax peptide (LLFGYPVYV) complex.

10 One or both of the high affinity TCR chains may be labelled with a detectable label, for example a label that is suitable for diagnostic purposes. Thus, the invention provides a method for detecting a TCR ligand selected from CD1-antigen complexes, bacterial superantigens, and MHC-peptide/superantigen complexes which method comprises contacting the TCR ligand with a high affinity TCR or multimeric high
15 affinity TCR complex in accordance with the invention which is specific for the TCR ligand; and detecting binding of the high affinity TCR or multimeric high affinity TCR complex to the TCR ligand. In tetrameric high affinity TCR complexes formed using biotinylated heterodimers, fluorescent streptavidin (commercially available) can be used to provide a detectable label. A fluorescently-labelled tetramer is suitable for use
20 in FACS analysis, for example to detect antigen presenting cells carrying the peptide for which the high affinity TCR is specific.

Another manner in which the soluble high affinity TCRs of the present invention may be detected is by the use of TCR-specific antibodies, in particular monoclonal
25 antibodies. There are many commercially available anti-TCR antibodies, such as α F1 and β F1, which recognise the constant regions of the α and β chain, respectively.

The high affinity TCR (or multivalent complex thereof) of the present invention may alternatively or additionally be associated with (e.g. covalently or otherwise linked to)
30 a therapeutic agent which may be, for example, a toxic moiety for use in cell killing, or an immunostimulating agent such as an interleukin or a cytokine. A multivalent

high affinity TCR complex of the present invention may have enhanced binding capability for a TCR ligand compared to a non-multimeric wild-type or high affinity T cell receptor heterodimer. Thus, the multivalent high affinity TCR complexes according to the invention are particularly useful for tracking or targeting cells presenting particular antigens *in vitro* or *in vivo*, and are also useful as intermediates for the production of further multivalent high affinity TCR complexes having such uses. The high affinity TCR or multivalent high affinity TCR complex may therefore be provided in a pharmaceutically acceptable formulation for use *in vivo*.

10 The invention also provides a method for delivering a therapeutic agent to a target cell, which method comprises contacting potential target cells with a high affinity TCR or multivalent high affinity TCR complex in accordance with the invention under conditions to allow attachment of the high affinity TCR or multivalent high affinity TCR complex to the target cell, said high affinity TCR or multivalent high affinity TCR complex being specific for the TCR ligand and having the therapeutic agent associated therewith.

In particular, the soluble high affinity TCR or multivalent high affinity TCR complex can be used to deliver therapeutic agents to the location of cells presenting a particular antigen. This would be useful in many situations and, in particular, against tumours. A therapeutic agent could be delivered such that it would exercise its effect locally but not only on the cell it binds to. Thus, one particular strategy envisages anti-tumour molecules linked to high affinity T cell receptors or multivalent high affinity TCR complexes specific for tumour antigens.

25 Many therapeutic agents could be employed for this use, for instance radioactive compounds, enzymes (perforin for example) or chemotherapeutic agents (cis-platin for example). To ensure that toxic effects are exercised in the desired location the toxin could be inside a liposome linked to streptavidin so that the compound is released slowly. This will prevent damaging effects during the transport in the body and ensure

that the toxin has maximum effect after binding of the TCR to the relevant antigen presenting cells.

Other suitable therapeutic agents include:

- 5 • small molecule cytotoxic agents, i.e. compounds with the ability to kill mammalian cells having a molecular weight of less than 700 daltons. Such compounds could also contain toxic metals capable of having a cytotoxic effect. Furthermore, it is to be understood that these small molecule cytotoxic agents also include pro-drugs, i.e. compounds that decay or are converted under physiological conditions to release cytotoxic agents. Examples of such agents include cis-platin, 10 maytansine derivatives, rachelmycin, calicheamicin, docetaxel, etoposide, gemcitabine, ifosfamide, irinotecan, melphalan, mitoxantrone, sorfimer sodiumphotofrin II, temozolmide, topotecan, trimetreate glucuronate, auristatin E vincristine and doxorubicin;
- 15 • peptide cytotoxins, i.e. proteins or fragments thereof with the ability to kill mammalian cells. Examples include ricin, diphtheria toxin, pseudomonas bacterial exotoxin A, DNAase and RNAase;
- radio-nuclides, i.e. unstable isotopes of elements which decay with the concurrent emission of one or more of α or β particles, or γ rays. Examples 20 include iodine 131, rhenium 186, indium 111, yttrium 90, bismuth 210 and 213, actinium 225 and astatine 213; chelating agents may be used to facilitate the association of these radio-nuclides to the high affinity TCRs, or multimers thereof;
- prodrugs, such as antibody directed enzyme pro-drugs;
- immuno-stimulants, i.e. moieties which stimulate immune response. Examples 25 include cytokines such as IL-2, chemokines such as IL-8, platelet factor 4, melanoma growth stimulatory protein, etc, antibodies or fragments thereof, complement activators, xenogeneic protein domains, allogeneic protein domains, viral/bacterial protein domains and viral/bacterial peptides.
- 30 Soluble high affinity TCRs or multivalent high affinity TCR complexes of the invention may be linked to an enzyme capable of converting a prodrug to a drug. This

allows the prodrug to be converted to the drug only at the site where it is required (i.e. targeted by the sTCR).

5 A multitude of disease treatments can potentially be enhanced by localising the drug through the specificity of soluble high affinity TCRs. For example, it is expected that the high affinity HLA-A2-tax (LLFGYPVYV) specific A6 TCR disclosed herein may be used in methods for the diagnosis and treatment of HTLV-1.

10 Viral diseases for which drugs exist, e.g. HIV, SIV, EBV, CMV, would benefit from the drug being released or activated in the near vicinity of infected cells. For cancer, the localisation in the vicinity of tumours or metastasis would enhance the effect of toxins or immunostimulants. In autoimmune diseases, immunosuppressive drugs could be released slowly, having more local effect over a longer time-span while minimally affecting the overall immuno-capacity of the subject. In the prevention of
15 graft rejection, the effect of immunosuppressive drugs could be optimised in the same way. For vaccine delivery, the vaccine antigen could be localised in the vicinity of antigen presenting cells, thus enhancing the efficacy of the antigen. The method can also be applied for imaging purposes.

20 The soluble high affinity TCRs of the present invention may be used to modulate T cell activation by binding to specific TCR ligand and thereby inhibiting T cell activation. Autoimmune diseases involving T cell-mediated inflammation and/or tissue damage would be amenable to this approach, for example type I diabetes. Knowledge of the specific peptide epitope presented by the relevant pMHC is required for this use.

25

Medicaments in accordance with the invention will usually be supplied as part of a sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical composition may be in any suitable form, (depending upon the desired method of administering it to a patient). It may be provided

30

in unit dosage form, will generally be provided in a sealed container and may be provided

as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It may include a plurality of said unit dosage forms.

5 The pharmaceutical composition may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

10

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions). Suitable excipients for tablets or hard gelatine capsules include lactose, 15 maize starch or derivatives thereof, stearic acid or salts thereof. Suitable excipients for use with soft gelatine capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

20

For the preparation of solutions and syrups, excipients which may be used include for example water, polyols and sugars. For the preparation of suspensions oils (e.g. vegetable oils) may be used to provide oil-in-water or water in oil suspensions.

25

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3(6):318 (1986). Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For infections of the eye or other external tissues, for example mouth and skin, the compositions are preferably applied as a topical ointment or cream.

30

When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be

formulated in a cream with an oil-in-water cream base or a water-in-oil base.

Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

Pharmaceutical compositions adapted for rectal administration may be presented as suppositories or enemas. Pharmaceutical compositions adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable compositions wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient. Pharmaceutical compositions adapted for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurised aerosols, nebulizers or insufflators. Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations. Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Excipients which may be used for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

Dosages of the substances of the present invention can vary between wide limits, depending upon the disease or disorder to be treated, the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used. The dosage may be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be reduced, in accordance with normal clinical practice.

The invention also provides a method for obtaining a high affinity TCR chain, which method comprises incubating such a host cell under conditions causing expression of the high affinity TCR chain and then purifying the polypeptide.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

Examples

The invention is further described in the following examples, which do not limit the scope of the invention in any way.

Reference is made in the following to the accompanying drawings in which:

Figures 1a and 1b show respectively the nucleic acid sequences of a soluble A6 TCR α and β chains, mutated so as to introduce a cysteine codon. The shading indicates the introduced cysteine codons.

5 Figure 2a shows the A6 TCR α chain extracellular amino acid sequence, including the $T_{48} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond, and Figure 2b shows the A6 TCR β chain extracellular amino acid sequence, including the $S_{57} \rightarrow C$ mutation (underlined) used to produce the novel disulphide inter-chain bond.

10

Figure 3 Outlines the cloning of TCR α and β chains into phagemid vectors. The diagram describes a phage display vector. RSB is the ribosome-binding site. S1 or S2 are signal peptides for secretion of proteins into periplasm of *E. coli*. The * indicates translation stop codon. Either of the TCR α chain or β chain can be fused to phage coat protein, however in this diagram only TCR β chain is fused to phage coat protein.

15

Figure 4 details the DNA sequence of phagemid pEX746:A6.

20

Figure 5 expression of phage particle fusions of bacterial coat protein and heterodimeric A6 TCR in *E. coli*. Fusion proteins of heterodimeric A6 TCR::gIII are detected using western blotting. Phage particles are prepared from *E. coli* XL-1-Blue and concentrated with PEG/NaCl. The samples are loaded in reducing or non-reducing sample buffers. Lane 1 is the sample of clone 1 containing correct sequence, and lane 2 is the sample of clone 2 containing a deletion in the α -chain encoding gene. The heterodimeric A6 TCR:gIII fusion protein was detected at 125kDa.

25

Figure 6 illustrates ELISA detection of pMHC peptide complex binding activity of a heterodimeric A6 TCR displayed on phage. Clone 1 binds specifically to HLA A2-Tax-complex. Clone 2 cannot bind to any pMHC, as no TCR is attached to the phage particles.

30

Figure 7a schematic illustration of the single-chain A6 TCR-C-Kappa DNA ribosome display construct.

- 5 Figure 7b details the complete DNA and amino acid sequences of the single-chain A6 TCR-C-Kappa DNA ribosome display construct encoded in pUC19.

Figure 8 details the DNA sequence of pUC19-T7.

- 10 Figure 9 details the DNA sequence of the single-chain A6 TCR-C-Kappa ribosome display construct that was cloned into pUC19-T7.

Figure 10 Western blot showing the detection of in-vitro translated single-chain A6 TCR-C-Kappa using Ambion rabbit reticulocyte lysates.

15

Figure 11 RT-PCR of the single-chain A6 TCR-C-Kappa mRNA on beads rescued from the ribosome display reactions.

- 20 Figure 12a details the DNA sequence of the A6 TCR Clone 9 mutated β chain; the mutated nucleic acid is indicated in bold.

Figure 12b details the amino acid sequence of the A6 TCR Clone 9 mutated β chain, the position corresponding to the introduced opal stop codon is indicated with an *.

- 25 Figure 13 details the DNA sequence of the A6 TCR Clone 49 mutated β chain; the mutated nucleic acid is indicated in bold. As this is a 'silent' mutation no change is introduced into the resulting amino acid sequence by this mutation.

- 30 Figure 14a details the DNA sequence of the A6 TCR Clone 134 mutated A6 TCR β chain; the mutated nucleic acids are indicated in bold.

Figure 14b details the amino acid sequence of the A6 TCR Clone 134 mutated A6 TCR β chain as tested by BIAcore assay; the mutated amino acids are indicated in bold.

5 Figure 14c details the amino acid sequence of the A6 TCR Clone 134 mutated A6 TCR β chain as tested by phage ELISA assay; the mutated amino acids are indicated in bold.

10 Figure 15 BIAcore data for the binding of A6 TCR clone 134 to HLA-A2 Tax and HLA-A2 NY-ESO

Figure 16 BIAcore data used to determine T_{OFF} for the binding of A6 TCR clone 134 to HLA-A2 Tax

15 Figures 17a and 17b show the DNA sequence of the mutated α and β chains of the NY-ESO TCR respectively

Figures 18a and 18b show the amino acid sequences of the mutated α and β chains of the NY-ESO TCR respectively

20 Figure 19 details the DNA and amino acid sequence of the NY-ESO TCR β chain as incorporated into the pEX746:NY-ESO phagemid respectively.

25 Figure 20 shows the specific binding of phage particles displaying the NY-ESO TCR to HLA-A2-NY-ESO in a phage ELISA assay.

Example 1 – Design of primers and mutagenesis of A6 Tax TCR α and β chains to introduce the cysteine residues required for the formation of a novel inter-chain disulphide bond

- 5 For mutating A6 Tax threonine 48 of exon 1 in TRAC*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C ACA GAC AAA tgT GTG CTA GAC AT

5'-AT GTC TAG CAC Aca TTT GTC TGT G

10

For mutating A6 Tax serine 57 of exon 1 in both TRBC1*01 and TRBC2*01 to cysteine, the following primers were designed (mutation shown in lower case):

5'-C AGT GGG GTC tGC ACA GAC CC

15 5'-GG GTC TGT Gca GAC CCC ACT G

PCR mutagenesis:

Expression plasmids containing the genes for the A6 Tax TCR α or β chain were mutated using the α -chain primers or the β -chain primers respectively, as follows.

- 20 100 ng of plasmid was mixed with 5 μ l 10 mM dNTP, 25 μ l 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted to 240 μ l with H₂O. 48 μ l of this mix was supplemented with primers diluted to give a final concentration of 0.2 μ M in 50 μ l final reaction volume. After an initial
- 25 denaturation step of 30 seconds at 95°C, the reaction mixture was subjected to 15 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs).
- 30 10 μ l of the digested reaction was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K₂HPO₄, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep

column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University. The respective mutated nucleic acid and amino acid sequences are shown in Figures 1a and 2a for the α chain and Figures 1b and 2b for the β chain.

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Example 2 – Construction of phage display vectors and cloning of A6 TCR α and β chains into the phagemid vectors.

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In order to display a heterodimeric A6 TCR containing a non-native disulfide inter-chain bond on filamentous phage particles, phagemid vectors were constructed for expression of fusion proteins comprising the heterodimeric A6 TCR containing a non-native disulfide inter-chain bond with a phage coat protein. These vectors contain a pUC19 origin, an M13 origin, a bla (Ampicillin resistant) gene, Lac promoter/operator and a CAP-binding site. The design of these vectors is outlined in Figure 3, which describes vectors encoding for both the A6 TCR β chain-gp3 or A6 TCR β chain-gp8 fusion proteins in addition to the soluble A6 TCR α chain. The expression vectors containing the DNA sequences of the mutated A6 TCR α and β chains incorporating the additional cysteine residues required for the formation of a novel disulfide inter-chain bond prepared in Example 1 and as shown in figures 1a and 1b were used as the source of the A6 TCR α and β chains for the production of a phagemid encoding this TCR. The complete DNA sequence of the phagemid construct (pEX746) utilised is given in Figure 4.

25

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in table-1 are used for construction of the vectors. An example of the PCR programme is 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 5 seconds, 53°C for 5 seconds and 72°C for 90 seconds, followed by 1 cycle of 72°C for 10 minutes, and then hold at 4°C. The Expand high fidelity Taq DNA polymerase is purchased from Roche.

30

5

Table 1. Primers used for construction of the A6 TCR phage display vectors

Primer name	Sequence 5' to 3'	Usage
	TAATAATACGTATAATAATATTCTATTTC AAG	pEX746 construction, SnaB1/
YOL1	GAGACAGTC	clone SDII
	CAATCCAGCGGCTGCCGTAGGCAATAGGTATT	pEX746 construction, clone
YOL2	TCATTATGACTGTCTCCTTGAAATAG	s1
	CtaCGGCAGCCGCTGGATTGTTATTACTCGCG	
YOL3	GCCCAGCCGGCCATGGCccag	Clone S1
	GTTCTGCTCCACTTCCTTCTGGGCCATGGCCG	
YOL4	GCTGGGCCG	Fuse S1 and A6 V α
YOL5	CAGAAGGAAGTGGAGCAGAAC	Clone A6 V α
	CTTCTTAAAGAATTCTTAATTAACCTAGGTTA	
YOL6	TTAGGAACCTTCTGGGCTGGGGAAG	Clone A6 C α
	GTTAATTAAGAATTCTTTAAGAAGGAGATATA	
YOL7	CATATGAAAAAATTATTATTCGCAATTC	Clone SDIII
	CGCGCTGTGAGAATAGAAAGGAACAACATAAG	
YOL8	GAATTGCGAATAATAATTTTTTCATATG	Clone S2
	CTTCTATTCTCACAGCGCGCAGGCTGGTGTC	
YOL9	ACTCAGAC	Clone S2 and A6 V β
	ATGATGTCTAGATGCGGCCGCTCTGCTCTAC	
YOL10	CCCAGGCCTC	Clone A6 C β
	GCATCTAGACATCATCACCATCATCACTAGAC	Clone (His) ₆ tag and amber
YOL11	TGTTGAAAGTTGTTTAGCAAAAC	codon and fuse to gIII
	CTAGAGGGTACCTTATTAAGACTCCTTATTAC	
YOL12	GCAGTATG	Clone gIII

Example 3 – Expression of fusions of bacterial coat protein and heterodimeric A6 TCR in E. coli.

In order to validate the construct made in Example 2, phage particles displaying the heterodimeric A6 TCR containing a non-native disulfide inter-chain bond were prepared using methods described previously for the generation of phage particles displaying antibody scFvs (Li *et al*, 2000, Journal of Immunological Methods 236: 133-146) with the following modifications. *E. coli* XL-1-Blue cells containing pEX746:A6 phagemid (i.e. the phagemid encoding the soluble A6 TCR α chain and an A6 TCR β chain fused to the phage gIII protein produced as described in Example 2) were used to inoculate 5 ml of Lbatg (Lennox L broth containing 100 μ g/ml of ampicillin, 12.5 μ g/ml tetracycline and 2% glucose), and then the culture was incubated with shaking at 37°C overnight (16 hours). 50 μ l of the overnight culture was used to inoculate 5 ml of TYPatg (TYP is 16g/l of peptone, 16g/l of yeast extract, 5g/l of NaCl and 2.5g/l of K₂HPO₄), and then the culture was incubated with shaking at 37°C until OD_{600nm} = 0.8. Helper phage M13 K07 was added to the culture to the final concentration of 5 X 10⁹ pfu/ml. The culture was then incubated at 37°C stationary for thirty minutes and then with shaking at 200 rpm for further 30 minutes. The medium of above culture was then changed to TYPak (TYP containing 100 μ g/ml of ampicillin, 25 μ g/ml of kanamycin), the culture was then incubated at 25°C with shaking at 250 rpm for 36 to 48 hours. The culture was then centrifuged at 4°C for 30 minutes at 4000 rpm. The supernatant was filtrated through a 0.45 μ m syringe filter and stored at 4°C for further concentration or analysis.

The fusion protein of filamentous coat protein and heterodimeric A6 TCR containing a non-native disulfide inter-chain bond was detected in the supernatant by western blotting. Approximately 10¹¹ cfu phage particles were loaded on each lane of an SDS-PAGE gel in both reducing and non-reducing loading buffer. Separated proteins were primary-antibody probed with an anti-M13 gIII mAb, followed by a second antibody conjugated with Horseradish Peroxidase (HRP). The HRP activity was then detected with Opti-4CN substrate kit from Bio-Rad (Figure 5). Theses data indicated that

disulfide-bonded A6 TCR of clone 1 is fused with filamentous phage coat protein, gIII protein.

5 *Example 4 – Detection of functional heterodimeric A6 TCR containing a non-native disulfide inter-chain bond on filamentous phage particles*

The presence of functional (HLA-A2-tax binding) A6 TCR displayed on the phage particles was detected using a phage ELISA method.

10 *TCR-Phage ELISA*

Binding of the A6 TCR-displaying phage particles to immobilised peptide-MHC in ELISA is detected with primary rabbit anti-fd antisera (Sigma) followed by alkaline phosphatase (AP) conjugated anti-Rabbit mAb (Sigma). Non specific protein binding sites in the plates can be blocked with 2% MPBS or 3% BSA-PBS

15 *Materials and reagents*

1. Coating buffer, PBS
2. PBS: 138mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄
3. MPBS , 3% marvel-PBS
- 20 4. PBS-Tween: PBS, 0.1% Tween-20
5. Substrate solution, Sigma FAST pNPP, Cat# N2770

Method

1. Rinse NeutrAvidin coated wells twice with PBS.
- 25 2. Add 25µl of biotin-HLA-A2 Tax or biotin-HLA-A2 NYESO in PBS at concentration of 10 µg/ml, and incubate at room temperature for 30 to 60 min.
3. Rinse the wells twice with PBS
4. Add 300 µl of 3% Marvel-PBS, and incubate at room temperature for 1hr. Mix the TCR-phage suspension with 1 volume of 3% Marvel-PBS and incubate at room temperature.
- 30 5. Rinse the wells twice with PBS

6. Add 25 μ l of the mixture of phage-A6 TCR/Marvel-PBS, incubate on ice for 1hr
7. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
- 5 8. Add 25 μ l of ice cold rabbit anti-fd antibody diluted 1:1000 in Marvel-PBS, and incubate on ice for 1hr
9. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
- 10 10. Add 25 μ l of ice cold anti-rabbit mAb-Ap conjugate diluted 1:50,000 in Marvel-PBS, and incubate on ice for 1hr
11. Rinse the wells three times with ice-cold PBStween, and three times with ice-cold PBS.
12. Add 150 μ l of Alkaline phosphatase yellow to each well and read the signal at 405nm

15 The results presented in Figure 6 indicate clone 1 produced a phage particle displaying an A6 TCR that can bind specifically to its cognate pMHC. (HLA-A2 Tax)

Analysis of the DNA sequence of this displayed A6 TCR revealed the presence of an 'opal' stop codon in the TCR β chain not present in the corresponding sequence of the expression vector construct of Example 2. This codon is 'read-through' with low frequency by ribosomes of the *E.coli* strain utilised resulting in the insertion of a tryptophan residue at this site and a much-reduced overall level of full-length β chain expression. From this observation it was inferred that only cells expressing this mutated A6 TCR sequence had survived the culture rounds of Example 3, and that therefore the high levels of A6 TCR predicted to be expressed by the original expression vector were toxic to the host cells.

Example 5 – single- chain TCR (scTCR) ribosome display

30 *Construction of Ribosome display scTCR vectors for use in generation of ribosome display PCR templates.*

Ribosome display constructs were cloned into the readily available DNA plasmid pUC19 in order to generate an error free and stable DNA PCR template from which to

conduct subsequent ribosome display experiments. Vector construction was undertaken in two steps so as to avoid the use of large oligonucleotide primers (with their associated error problems). The final A6 scTCR-C-Kappa DNA ribosome display construct is shown in a schematic form in figure 7a and both DNA and protein ' sequences are shown in Figure 7b. This construct can be excised from pUC19 as a Pst1/EcoR1 double digest.

The molecular cloning methods for constructing the vectors are described in "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell". Primers listed in Table 2 are used for construction of the vectors. The PCR programme utilised was as follows – 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 30 seconds, 55°C for 20 seconds and 72°C for 120 seconds, followed by 1 cycles of 72°C for 5 minutes, and then hold at 4°C. The Pfu DNA polymerase is purchased from Strategene. Oligonucleotide primers used are described in table 2.

Construction of pUC19-T7- Step 1

The construction of pUC19-T7 is described below, the construction results in a pUC19 vector containing a T7 promoter region followed by a short space region and the an optimum eukaryotic Kozak sequence. This is an essential part of the ribosome display construct as it is required for the initiation of transcription of any attached sequence in rabbit reticulocyte lysates. Sequences for ribosome display such as the A6scTCR-Ckappa can be ligated into the pUC19-T7 vector between the Nco1 and EcoR1 restriction sites.

Equimolar amounts of the primer Rev-link and For-link were annealed by heating to 94°C for 10 min and slowly cooling the reaction to room temperature. This results in the formation of a double stranded DNA complex that can be seen below.

5 ' AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG
 CGTCGATTATGCTGAGTGATATCCTTGTCCGGTGGTACCCTAG 3 '

5 The 5' region contains an overhanging sticky end complimentary to a HindIII restriction site whilst the 3' end contains a sticky end that is complimentary to a BamHI restriction site.

The annealed oligonucleotides were ligated into Hind III/BamHI double-digested pUC19 which had been purified by agarose gel electrophoresis, excised and further
 10 purified with the Qiagen gel extraction kit. The ligations were transformed into *E. coli* XL1-BLUE. Individual pUC19-T7 clones were sequenced to confirm the presence of the correct sequence. The sequence is shown in Figure 8.

Construction of A6scTCR-C-Kappa vector – Step 2.

15

Construction of the single chain A6scTCR-C-Kappa DNA sequence requires the generation of three PCR fragments that must then be assembled into one A6scTCR-C-Kappa fragment. The fragments consist of (a.) the A6 TCR alpha chain variable region flanked by a NcoI site in the 5' region and a section of Glycine Serine linker in the 3' region flanked by a BamHI restriction site. This product was generated via a standard
 20 PCR of the vector pEX202 with the primers 45 and 50 (See Table 2). Fragment (b.) A6 TCR beta variable and constant region flanked by a BamHI restriction site in the 5' region followed by a section of Glycine Serine linker. This product was generated via a standard PCR of the vector pEX207 with the primers 72 and 73 (See Table 2).
 25 Fragment (c.) Portion of a human C-kappa region generated by a standard PCR of the p147 vector with the primers 61-60 (See Table 2). All PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.

Fragments (b.) and (c.) were fused by a standard overlap PCR via the complementarity
 30 in their primer sequences 73 and 61 (See Table 2). The PCR was carried out via the primers 72 and 60 (See Table 2). The PCR products were run on a 1.6% TBE agarose

gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit. This fragment is termed (d.).

Fragment (a.) was double digested with Nco1 and BamH1 whilst fragment (d.) was double digested with BamH1 and EcoR1. pUC19-T7 was double digested with Nco1 and EcoR1. All digested DNA products were run on a 1.2% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit. The digested pUC19-T7, fragments (a.) and (d.) were ligated and transformed into E. coli XL1-BLUE. Transformants were sequenced to confirm the correct sequence. The sequence of the A6scTCR-C-Kappa ribosome display construct that was cloned into pUC19 is shown in Figure 9 flanked by its Pst1 and EcoR1 sites.

Table 2.

Oligonucleotides used (Purchased from MWG).

Rev-Link	5' GATCCCATGGTGGCCTGTTCTATAGTGAGTCGTATTAGCTGC
For-Link	5' AGCTGCAGCTAATACGACTCACTATAGGAACAGGCCACCATGG
45-A6	5' CCACCATGGGCCAGAAGGAAGTGGAGCAGAACTC
7 A6-Beta(RT-PCR)(a)	5' CGAGAGCCCGTAGAACTGGACTTG
49-A6-BamH1-F	5' GTGGATCCGGCGGTGGCGGGTCGAACGCTGGTGTCA CTCAGACCCC
50-A6-BamH1-R	5' CCGGATCCACCTCCGCCTGAACCGCCTCCACCGGTGACCACAAC CTGGGTCCCTG
60-Kappa-rev-EcoR1	5' CTGAGAATTCTTATGACTCTCCGCGGTTGAAGCTC
61-Betac-Kappa-for1	5' TGACGAATTCTGACTCTCCGCGGTTGAAGCTC
71 T7-Primer	5' AGCTGCAGCTAATACGACTCACTATAGG
72 A6-beta	5' GGCCACCATGGGCAACGCTGGTGTCACTCAGACCCC
73-A6-cons-rev	5' TGAACCGCCTCCACCGTCTGCTCTACCCAGGCCTCGGCG
75 Kappa-rev	5' TGACTCTCCGCGGTTGAAGCTC

Demonstration of the production of sc A6 TCR-C-Kappa by In vitro transcription translation.

Preparation of scA6 TCR-C-Kappa PCR product for In vitro transcription translation.

5

Here we describe the synthesis of sc A6 TCR-C-Kappa via *In vitro* transcription translation in the presence of biotinylated lysine and its subsequent detection by western blotting and detection with alkaline phosphatase labelled streptavidin.

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The sc A6 TCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector sc A6 TCR-C-Kappa as template and PCR primers 71 and 60. Primer 60 contains a stop codon to allow the release of the scTCR from the ribosome. Pfu polymerase (Stratagene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size excised and purified using the Qiagen gel extraction kit.

15

The transcription translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit Cat 1280-1287 with 300ng of the above described PCR product. Three transcription translation reactions were set up according to the manufactures protocol. The one modification was the addition of biotinylated lysine from the Transcend™ Non-Radioactive Translation Detection System.

20

Reaction 1 sc A6 TCR-C-Kappa 300ng with 2µl biotinylated lysine

25

Reaction 2 sc A6 TCR-C-Kappa 300ng without 2µl biotinylated lysine

Reaction 3 No DNA control with 2µl biotinylated lysine.

30

Two microliters of each reaction was run on a 4-20% Novex gradient SDS-PAGE gel (Invitrogen). Additionally a number of dilutions of a control biotinylated TCR were also run. The gel was blotted and the proteins detected with streptavidin alkaline phosphatase and subsequently colometrically developed with Western Blue ® Stabilized Substrate for Alkaline Phosphatase as described in the Transcend™ Non-

Radioactive Translation Detection System protocol. The western blot is shown in Figure 10.

In the no DNA control and A6scTCR-C-Kappa reaction without biotinylated lysine no band of approximately the correct size can be seen as expected whilst in the A6scTCR-C-Kappa reaction in the presence of biotinylated lysine a band of approximately the correct size can be seen. This demonstrates the synthesis of the sc A6 TCR-C-Kappa TCR by *In vitro* transcription translation.

Preparation of sc A6 TCR-C-Kappa ribosome display PCR product.

The sc A6 TCR-C-Kappa PCR product was prepared in a standard PCR reaction using the vector A6scTCR-C-Kappa as template and PCR primers 71 and 75 (See Table 2). Primer 75 does not contain a stop codon. Pfu polymerase (Stratagene) was used for increased fidelity during PCR synthesis. The PCR products were run on a 1.6% TBE agarose gel and DNA bands of the correct size were excised and purified using the Qiagen gel extraction kit.

Ribosome Display Process

Transcription and translation of sc A6 TCR-C-Kappa

The transcription / translation reactions were carried out using the Ambion PROTEINscript II Linked transcription translation kit (Cat No. 1280-1287)

Transcription reactions

The following transcription reactions were set up in Ambion 0.5 ml non stick tubes (Cat No. 12350).

Contents	tube 1 (Normal A6)	2 (Control)
Water	4.53 µl	5.7µl
Template (PCR product)	Sc A6 TCR-C-Kappa PCR product 1.17µl (300 ng)	No DNA

5X transcription mix	2 μ l	2 μ l
Enzyme mix	2 μ l	2 μ l
Supersasin <small>Rnase inhibitor</small> <small>ambion</small>	0.3 μ l	0.3 μ l
Final volume	10 μ l	10 μ l

The tubes were incubated at 30°C for 60 min on a PCR block with the hot lid off.

Translation reactions

- 5 The following translation reactions were set up in Ambion 0.5 ml non stick tubes.

Contents	1 (Normal A6)	2 (Control)
Reticulocyte Lysate	105 μ l	105 μ l
25mM Mg-Acetate	3 μ l	3 μ l
Translation Mix	7.5 μ l	7.5 μ l
Methionine	7.5 μ l	7.5 μ l
Water	18 μ l	18 μ l
Supersasin <small>Rnase inhibitor</small>	3 μ l	3 μ l
Transcription reaction	6 μ l tube 1 above	6 μ l tube 2 above

- 10 Each tube contains enough for 3x50 μ l selections. The tubes were mixed and incubated at 30°C for 60 min on a PCR block with the hot lid off. After 30 min 3 Unit of RQ1 Rnase free Dnase (Promega) was added to destroy the original DNA template in tube 1 and 3 Unit RQ1 Rnase free Dnase (Promega) in tube 2. After 60 min 18 μ l of Heparin solution was added to translation reaction 2 and 18 μ l of Heparin solution was added to translation reaction 1. Samples were stored on ice ready for selection against HLA-
- 15 coated beads.

Coating of magnetic beads.

20µl of resuspended Streptavidin Magnetic Particles (Roche Cat. No. 1641778) were transferred into a sterile Rnase free 1.5 ml eppendorf tube. The beads were immobilised with a Magnetic Particle Separator (Roche Cat. No. 1641794) and the supernatant was removed. The beads were then washed with 100 µl of Rnase free 1 X PBS (10 x PBS Ambion Cat No. 9624, Ambion H₂O Cat No. 9930) the beads were immobilised and the supernatant was removed. A total of 3 PBS washes were carried out.

The beads were resuspended in 20µl of PBS and the contents split evenly between two tubes (10µl each). One tube will be used to produce control-blocked beads and the other tubes to produce HLA-A2-Tax coated beads.

To the control beads tube 80µl of BSA/Biotin solution was added and mixed. The BSA/Biotin solution was made up as follows. 10µl of a 0.2M Tris base 0.1M Biotin solution was added to 990µl of PBS 0.1 % BSA (Ambion Ultrapure Cat No. 2616). Also 20 µl of Heparin solution (138 mg/ml Heparin (Sigma H-3393) in 1 x PBS) was added and the solution mixed. The beads were incubated at room temperature for 1 hour with intermittent mixing. The beads were then washed three times with 100µl of PBS and were resuspended in 10 µl of PBS, 0.1% BSA.

The HLA-TAX coated beads were prepared as follows. 40 µl of HLA-A2-Tax(1.15 mg/ml prepared as described in WO99/60120) was added to the 10 µl of beads and mixed. The beads were incubated at room temperature for 15 min and then 20 µl of BSA 50mg/ml Ambion Cat 2616 and 20 µl of heparin solution (see above) were added and mixed. The beads were incubated for a further 45 min and then 20 µl of BSA/Biotin solution was added. The beads were then washed three times with 100 µl of PBS and were re-suspended in 10 µl of PBS, 0.1% BSA.

Panning with magnetic beads

The sc A6 TCR translation reaction was split into three 50µl aliquots and each aliquot received either 2µl of the following beads:

- 5 Control (no HLA)
 HLA-A2-Tax
 HLA-A2-Tax plus 10µg soluble scA6 TCR

- 10 A control translation reaction was also carried out and split into three 50µl aliquots
 and each aliquot received either 2µl of the following beads

- Control (no HLA)
 HLA-A2-Tax
 HLA-A2-Tax plus 10µg soluble sc A6 TCR

- 15 This gave a total of six tubes. The tubes were incubated on a PCR block at 5°C for 60
 min with intermittent mixing.

- 20 The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-
 acetate, 0.2% Tween 20(Sigma Rnase free). Each aliquot of beads were then re-
 suspended in 50µl of 1 x RQ1 Dnase digestion buffer containing 1µl (40 U) of
 Supersasin and 1µl (1U) of RQ1 Dnase. The beads were incubated on a PCR block for
 30 min at 30°C.

- 25 The beads were then washed three times with 100µl ice cold buffer (PBS, 5mM Mg-
 acetate, 0.2% Tween 20) and once with ice cold H₂O. The beads were re-suspended in
 10µl of Rnase free H₂O. The beads were then frozen ready for RT-PCR.

The RT PCR reactions on the beads were carried out using the Titan one tube RT-PCR kit cat 1855476 as described in the manufacturers protocols. Two microliters of beads were added into each RT-PCR reaction along with the primers 45 and 7 and 0.3µl of Superscript RNase inhibitor.

5 For each RT-PCR reaction a second PCR only reaction was set up which differed only by the fact that no reverse transcriptase was present just Roche high fidelity polymerase. This second reaction served as a control for DNA contamination. Additionally a RT-PCR positive control control was set up using 1ng of the vector sc A6 TCR-C-Kappa.

10

The reactions were cycled as follows. An RT-PCR step was carried out by incubation of the samples at 50°C for 30 min followed by the inactivation of the reverse transcriptase by incubation at 94°C for 3 min on a PCR block.

The reactions were then PCR cycles as follows for a total of 38 cycles.

15

94°C 30 seconds

55°C 20 seconds

68°C 130 seconds.

The PCR reaction was finished by incubation at 72°C for 4 minutes.

Great care was taken during all ribosome display steps to avoid RNase contamination.

20

The RT-PCR and PCR reactions were run on a 1.6% TBE agarose gel which can be seen in Figure 11. Analysis of the gel shows that there is no DNA contamination and that all PCR products are derived from mRNA. The DNA band of the correct size in lane 2 demonstrates that ribosome displayed sc A6 TCR-C-Kappa was selected out by HLA-A2-Tax coated beads. Lane 3 shows that we can inhibit this specific selection of

25 ribosome-displayed sc A6 TCR-C-Kappa by the addition of soluble sc A6 TCR. The significant reduction in the band intensity in lane 3 relative to the uninhibited sample in lane 2 demonstrates this. No binding of ribosome-displayed sc A6 TCR-C-Kappa could be shown against control non-HLA coated beads.

Example 6 – Sequence Analysis of A6 TCR clones displayed on phage particles and methods to improve display characteristics

After the construction of vectors for displaying A6 TCR on phage by PCR and molecular cloning, bacterial clones that can produce phage particles displaying A6 TCR were screened by phage ELISA as described in Example 4. Three different clones were identified that gave specific binding to HLA-A2-tax in the ELISA binding assay. These clones all contained mutations in the 'wild-type' A6 TCR DNA or in the associated regulatory sequences, which are described in the following table:

Functional clones from screening TCR A6 displayed on phage

Name	Feature	
Clone 7	The third ribosome-binding site, which is located in front of v β gene, is mutated from <u>AAGGAGA</u> to <u>AAGGGGA</u> .	
Clone 9	An opal codon is introduced in v β CDR3.	Full DNA and amino acid sequence in Figures 12a & 12b
Clone 49	An amber codon is introduced in v β FR1. This mutation introduces a 'silent' mutation that does not affect the resulting amino acid sequence	Full DNA sequence in Figures 13a

These clones all contained mutations that are likely to cause a reduction in the expression levels of the A6 TCR β chain. It was inferred that low expression clones were selected over high expression clones as a result of cell toxicity caused by high expression levels of TCR.

Example 7– Mutagenesis of A6 TCR CDR3 regions



5

PCR was used to introduce mutations for affinity maturation. The A6 TCR clone 9 (incorporating an introduced opal codon in the β chain CDR3 sequence) was used as a source of template DNA, and TCR chains were amplified with the mutation primers (detailed in the following table) and YOL22 5'CATTTCAGGGATAGCAAGC3' (β -chains) or YOL13 5'TCACACAGGAAACAGCTATG3' (α -chains).

15



Primer		
name	Sequence 5' to 3'	Usage
	TGTGCCTCGAGGNNKNNKNNKNNKNNKN	A6 beta CDR3 library1 96-101
YOL59	NKCGACCAGAGCAGTACTTCG	forward
	TGTGCCTCGAGGCCGNNKNNKNNKNNKN	A6 beta CDR3 library2 97-102
YOL60	NKNNKCCAGAGCAGTACTTCGGGC	forward
	TGTGCCTCGAGGCCGNNKNNKNNKNNKN	A6 beta CDR3 library3 97-
YOL61	NKNNKCGACCAGAGCAGTACTTCG	99,insert1,100,101 forward
	TGTGCCTCGAGGCCGNNKNNKNNKNNKN	A6 beta CDR3 library4 97-
YOL62	NKNNKGGAGGGCGACCAGAGCAG	99,insert3,forward
	TGTGCCTCGAGGCCGGGANNNKNNKNNKN	A6 beta CDR3 library5 98-
YOL63	NKNNKNNKGGGCGACCAGAGCAGTAC	99,insert3,100 forward
	TGTGCCTCGAGGNNKNNKNNKNNKNNKN	A6 beta CDR3 library5 98-
YOL68	NKCCAGAGCAGTACTTCGggc	99,delete1 forward
	TGTGCCTCGAGGNNKNNKNNKNNKNNKN	A6 beta CDR3 library6 98-
YOL69	NKGAGCAGTACTTCGggccg	99,delete2 forward
	TGTGCCTCGAGGNNKNNKNNKNNKNNKN	A6 beta CDR3 library7 98-
YOL70	NKCAGTACTTCGggccgggc	99,delete3 forward
	TGTGCCTCGAGGccgNNKNNKNNKNNKg	
YOL71	ggCGACCAGAGCAGTACTTCG	A6 beta CDR3 library8 center4
	AAACTGAAGCTTNNNNNNNNNNNNNNNT	
YOL58	GTAACGGCACAGAGGTAG	A6 alpha CDR3 library1 reverse

	AAACTGAAGCTTMNNMNNgctgtcMNNT	A6 alpha CDR3 library2 reverse
YOL72	GTAACGGCACAGAGGTAG	conserve DS
	AAACTGAAGCTTMNNMNNMNNgctgtcM	A6 alpha CDR3 library3 reverse
YOL73	NNTGTAAACGGCACAGAGGTAG	conserve DS insert1
	AAACTGAAGCTTMNNMNNgctgtcMNNA	A6 alpha CDR3 library4 reverse
YOL74	ACGGCACAGAGGTAG	conserve DS deletel

α -chain fragments were digested with *Nco I* and *HindIII* and re-purified using a Qiagen kit and vector was prepared by digesting clone 9 with *Nco I* and *HindIII* followed by gel purification using a Qiagen kit. β -chain fragments were digested with *Xho I* and *Not I* and re-purified using a Qiagen kit and vector was prepared by digesting clone 9 with *Xho I* and *Not I* followed by gel purification using a Qiagen kit. Purified inserts and vectors at 3:1 molar ratio were mixed with T4 ligase buffer, T4 ligase and nuclease-free water. The ligations were carried out at 16°C water bath overnight. For each mutation-library, a total of 0.5 to 1µg purified ligated products were electroporated into *E. coli* TG1 at ratio of 0.2µg DNA per 40 µl of electroporation-competent cells (Stratagen) following the protocols provided by the manufacturer. After electroporation, the cells were re-suspended immediately with 960µl of SOC medium at 37°C and plated on a 244mm x244mm tissue culture plate containing YTE (15g Bacto-Agar, 8g NaCl, 10g Tryptone, 5g Yeast Extract in 1 litre) supplemented with 100µg/ml ampicillin and 2% glucose. The plate was incubated at 30°C over night. The cells were then scraped from the plates with 5 ml of DYT (16g Tryptone, 10g Yeast extract and 5g NaCl in 1 litre, autoclaved at 125°C for 15 minutes) supplemented with 15% glycerol.

In order to make phage particles displaying the A6 TCR, 500 ml of DYT_{Tag} (DYT containing 100 µg/ml of ampicillin and 2% glucose) was inoculated with 500 to 1000 µl of the library stocks. The culture was grown until OD(600nm) reached 0.5. 100 ml of the culture was infected with helper phage (M13 K07 (Invitrogen), or HYPER PHAGE (Progen Biotechnik, GmbH 69123 Heidelberg), and incubated at 37°C water bath for 30 minutes. The medium was replaced with 100 ml of DYT_{Tak} (DYT containing 100 µg/ml ampicillin and 25 µg/ml of kanamycin). The culture was then incubated with shaking at 300 rpm and 25°C for 20 to 36 hours.

Example 8 – Isolation of high affinity A6 TCR mutants

The isolation of high affinity A6 TCR mutants was carried out using two different methods.

The first method involves selecting phage particles displaying mutant A6 TCRs capable of binding to HLA-A2 Tax complex using Maxisorp immuno-tubes (Invitrogen). The immuno-tubes were coated with 1 to 2 ml 10 µg/ml streptavidin in PBS overnight at room temperature. The tubes were washed twice with PBS, and then 1 ml of biotinylated HLA-A2 Tax complex at 5 µg/ml in PBS was added and incubated at room temperature for 30 minutes. The rest of the protocol for selection of high affinity binders is as described previously (Li *et al.* (2000) *Journal of Immunological Methods* 236: 133-146), except for the following modifications. The selection was performed over three or four rounds. The concentrations of biotinylated HLA-A2 Tax complex were 5 µg/ml for the first round of selection, 0.5 µg/ml for the second, 0.05 µg/ml for the third and 0.005 µg/ml for the fourth round of selection. M13 K07 helper phage were used in rounds one and two, and hyper phage were used in subsequent rounds, for the selection.

The second method utilised was the selection of phage particles displaying mutant A6 TCRs capable of binding to HLA-A2 Tax complex in solution. Streptavidin-coated paramagnetic beads (Dynal M280) were pre-washed according to manufacturer's protocols. Phage particles, displaying mutated A6 TCR at a concentration of 10^{12} to 10^{13} cfu, were pre-mixed with biotinylated HLA-A2 Tax complex at concentrations of 2×10^{-8} M, 2×10^{-9} M, 2×10^{-10} M and 2×10^{-11} M for first, second, third and fourth-round of selections respectively. The mixture of A6 TCR-displaying phage particles and HLA-A2 Tax complex was incubated for one hour at room temperature with gentle rotation, and A6 TCR-displaying phage particles bound to biotinylated HLA-A2 Tax complex were captured using 200 µl (round 1) or 50 µl (round 2, 3, and 4) of streptavidin-coated M280 magnetic beads. After capture of the phage particles, the beads were washed a total of ten times (three times in PBStween20, twice in PBStween 20 containing 2%

skimmed milk powder, twice in PBS, once in PBS containing 2% skimmed milk powder, and twice in PBS) using a Dynal magnetic particle concentrator. After final wash, the beads were re-suspended in 1ml of freshly prepared 100mM triethylamine pH11.5, and incubated for 5 to 10 minutes at room temperature with gentle rotation.

- 5 Phage particles eluted from the beads were neutralized immediately with 300 μ l of 1M tris-HCl pH7.0. Half of the eluate was used to infect 10 ml of *E.coli* TG1 at OD(600nm)=0.5 freshly prepared for the amplification of the selected phage particles according to the methods previously described (Li *et al.*, (2000) *Journal of Immunological Methods* 236: 133-146).

10

After the third or fourth round of selection, 95 colonies were picked from the plates and used to inoculate 100 μ l of DYTag in a 96-well microtiter plate. The culture was incubated at 37°C with shaking overnight. 100 μ l of DYTag was then sub-inoculated with 2 to 5 μ l of the overnight cultures, and incubated at 37°C with shaking for 2 to 3

15 hours or until the culture became cloudy. To infect the cells with helper phage, the culture was infected with 25 μ l of DYTag containing 5×10^9 pfu helper phages, and incubated at 37°C for 30 minutes. The medium was replaced with DYTak. The plates were incubated at 25°C for 20 to 36 hours with shaking at 300 rpm. The cells were precipitated by centrifugation at 3000g for 10 minutes at 4°C. Supernatants were used

20 to screen for high affinity A6 TCR mutants by competitive phage ELISA as follows.

25

Nunc-Immuno Maxisorp wells coated with streptavidin were rinsed twice with PBS. 25 μ l 5 μ g/ml biotinylated HLA-A2-Tax complex was added to each well and these were incubated at room temperature for 30 to 60 minutes, and followed by two PBS rinses. Non-specific protein binding sites in the wells were blocked by the addition of 300 μ l 3% skimmed milk in PBS followed by incubation at room temperature for 2 hours. In order to prepare phage particles displaying the heterodimeric A6 TCR, phage particles were mixed with 3% skimmed milk in PBS containing 0, 20, and 200 nM HLA-A2-Tax, followed by incubation at room temperature for 1 hour. The phage is

30

added to the wells coated with HLA-A2-Tax and incubated at room temperature for 1

hour, followed by 3 washes with PBS containing 0.1% tween 20 and then 3 washes with PBS. The bound TCR-dispalying phage particles are detected with an anti-fd antibody (Sigma) as described in Example 4.

Several high affinity A6 TCR mutants were identified, and the CDR3 sequences are listed in the two following tables. Amber stop codons (*) were found in all β chain mutants and one α chain mutant.

10 A6 TCR β chain mutants

clone	CDR3 sequence
134	GCCTCGAGGCCGGGGCTGATGAGTGCGTAGCCAGAGCAGTAC A S R P G L M S A * P E Q Y
86	GCCTCGAGGCCGGGGCTGAGGTCGGCGTAGCCAGAGCAGTAC A S R P G L R S A * P E Q Y
87	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGGCGTAG A S R P G L A G G R P E A *
89	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGAGGATTAG A S R P G L A G G R P E D *
85	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGATCAGTAG A S R P G L A G G R P D Q *
83	GCCTCGAGGCCGGGTCTGTAGGCTGGGCGACCAGAGCAGTAC A S R P G L * A G R P E Q Y
1	GCCTCGAGGCCGGGGCTGGTTCGGGGCGACCAGAGCAGTAG A S R P G L V P G R P E Q *
2	GCCTCGAGGCCGGGGCTTGTGTCTGCTTAGCCAGAGCAGTAC A S R P G L V S A * P E Q Y
111	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCACATCCGTAG A S R P G L A G G R P H P *
125	GCCTCGAGGCCGGGACTAGCGGGAGGGCGACCAGATGCGTAG A S R P G L A G G R P D A *
133	GCCTCGAGGCCGGGTCTGATTAGTGCTTAGCCAGAGCAGTAC A S R P G L I S A * P E Q Y

A6 TCR α chain mutants

Clone	CDR3
149	GCCGTTACAAC TGACAGCTGGGGGCCGCTTCAG A V T T D S W G P L Q
65	GCCGTTACAAC TGACAGCTGGGGGAAGATGCAG A V T T D S W G K M Q
66	GCCGTTACAAC TGACAGCTGGGGGAAGTTGCAT A V T T D S W G K L H

153	GCCGTTACAACCTGACAGCTGGGGGTAGCTTCAT A V T T D S W G * L H
71	GCCGTTACAACCTGACAGCTGGGGGGAGCTTCAT A V T T D S W G E L H
70	GCCGTTACAACCTGACAGCTGGGGGAGGCTGCAT A V T T D S W G R L H
121	GCCGTTACAACCTGACAGCTGGGGGCAGCTTCAT A V T T D S W G Q L H
117	GCCGTTACAACCTGACAGCTGGGGGAAGGTTTCAT A V T T D S W G K V H
72	GCCGTTACAACCTGACAGCTGGGGGAAGGTGAAT A V T T D S W G K V N
150	GCCGTTACAACCTGACAGCTGGGGGAAGCTTCTG A V T T D S W G K L L

Example 9 – Production of soluble heterodimeric A6 TCR with non-native disulfide bond between constant regions, containing CDR3 mutations

5

Phagemid DNA encoding the high affinity A6 TCR mutants identified in Example 8 was isolated from the relevant *E.coli* cells using a Mini-Prep kit (Quiagen, UK)

10 PCR amplification using the phagemid DNA as a target and the following primers was used to amplify the soluble TCR α and β DNA sequences.

15 A6 TCR alpha chain forward primer
ggaattc atcgatg cagaaggaagtggagcag
ClaI restriction site is underlined)

20 Universal TCR alpha chain reverse primer
gtacacggccgggtcagggttctggatatac
(EagI restriction site is underlined)

25 A6 beta chain forward primer
tctctcattaatgaatgctggtgtcactcagacccc
(AseI restriction site is underlined)

Universal beta chain reverse primer
 tagaaaccggtggccagggcacaccagtgtggc
 (AgeI restriction site is underlined)

5 In the case of the TCR β chain a further PCR stitching was carried out to replace the
 amber stop codon in the CDR3 region with a codon encoding glutamic acid. When an
 amber stop codon is suppressed in *E.coli*, a glutamine residue is normally introduced
 instead of the translation being stopped. Therefore, when the amber codon-containing
 TCR is displayed on the surface of phage, it contains a glutamine residue in this
 10 position. However, when the TCR β -chain gene was transferred into the expression
 plasmid, a glutamic acid residue was used as an alternative to glutamine. The primers
 used for this PCR stitching were as follows.

15

YOL124 CTGCTCTGGTTCCGCACTC
 YOL125 GAGTGCAGGAACCAGAGCAG

The DNA sequence of the mutated soluble A6 TCR β chain was verified by automated
 sequencing (see Figure 14a for the mutated A6 TCR β chain DNA sequence and 14b
 for the amino acid sequence encoded thereby). Figure 14c shows the mutated A6 TCR
 20 β chain amino acid sequence without the glutamine to glutamic acid substitution, i.e.
 the sequence that was present in Clone 134 as isolated by phage-ELISA.

Theses A6 TCR α and β DNA sequences were then used to produce a soluble A6 TCR
 as described in WO 03/020763. Briefly, the two chains are expressed as inclusion
 25 bodies in separate *E.coli* cultures. The inclusion bodies are then isolated, de-natured
 and re-folded together *in vitro*.

*Example 10 – BIAcore surface plasmon resonance characterisation of a high affinity
 A6 TCR binding to HLA-A2 Tax.*

30

A surface plasmon resonance biosensor (BIAcore 3000™) was used to analyse the
 binding of the high affinity clone 134 A6 TCR (See Figures 15a & 15b for the full
 DNA and amino acid sequences of the mutated TCR β chain respectively) to the HLA-

A2 Tax ligand. This was facilitated by producing pMHC complexes (described below) which were immobilised to a streptavidin-coated binding surface in a semi-oriented fashion, allowing efficient testing of the binding of a soluble T-cell receptor to up to four different pMHC (immobilised on separate flow cells) simultaneously.

5 Manual injection of HLA complex allows the precise level of immobilised class I molecules to be manipulated easily.

Biotinylated class I HLA-A2 tax complexes were refolded *in vitro* from bacterially-expressed inclusion bodies containing the constituent subunit proteins and synthetic peptide, followed by purification and *in vitro* enzymatic biotinylation (O'Callaghan *et al.* (1999) *Anal. Biochem.* **266**: 9-15). HLA-heavy chain was expressed with a C-terminal biotinylation tag which replaces the transmembrane and cytoplasmic domains of the protein in an appropriate construct. Inclusion body expression levels of ~75 mg/litre bacterial culture were obtained. The HLA light-chain or β 2-microglobulin
10
15 was also expressed as inclusion bodies in *E.coli* from an appropriate construct, at a level of ~500 mg/litre bacterial culture.

E. coli cells were lysed and inclusion bodies were purified to approximately 80% purity. Protein from inclusion bodies was denatured in 6 M guanidine-HCl, 50 mM Tris pH 8.1, 100 mM NaCl, 10 mM DTT, 10 mM EDTA, and was refolded at a concentration of 30 mg/litre heavy chain, 30 mg/litre β 2m into 0.4 M L-Arginine-HCl, 100 mM Tris pH 8.1, 3.7 mM cystamine, mM cysteamine, 4 mg/ml peptide (e.g. tax 11-19), by addition of a single pulse of denatured protein into refold buffer at < 5°C. Refolding was allowed to reach completion at 4°C for at least 1 hour.

25 Buffer was exchanged by dialysis in 10 volumes of 10 mM Tris pH 8.1. Two changes of buffer were necessary to reduce the ionic strength of the solution sufficiently. The protein solution was then filtered through a 1.5 μ m cellulose acetate filter and loaded onto a POROS 50HQ anion exchange column (8 ml bed volume). Protein was eluted
30 with a linear 0-500 mM NaCl gradient. HLA-A2-peptide complex eluted at

approximately 250 mM NaCl, and peak fractions were collected, a cocktail of protease inhibitors (Calbiochem) was added and the fractions were chilled on ice.

5 Biotinylation tagged HLA-A2 complexes were buffer exchanged into 10 mM Tris pH 8.1, 5 mM NaCl using a Pharmacia fast desalting column equilibrated in the same buffer. Immediately upon elution, the protein-containing fractions were chilled on ice and protease inhibitor cocktail (Calbiochem) was added. Biotinylation reagents were then added: 1 mM biotin, 5 mM ATP (buffered to pH 8), 7.5 mM MgCl₂, and 5 µg/ml BirA enzyme (purified according to O'Callaghan *et al.* (1999) *Anal. Biochem.* 266: 9-15). The mixture was then allowed to incubate at room temperature overnight.

15 Biotinylated HLA-A2 complexes were purified using gel filtration chromatography. A Pharmacia Superdex 75 HR 10/30 column was pre-equilibrated with filtered PBS and 1 ml of the biotinylation reaction mixture was loaded and the column was developed with PBS at 0.5 ml/min. Biotinylated HLA-A2 complexes eluted as a single peak at approximately 15 ml. Fractions containing protein were pooled, chilled on ice, and protease inhibitor cocktail was added. Protein concentration was determined using a Coomassie-binding assay (PerBio) and aliquots of biotinylated HLA-A2 complexes were stored frozen at -20°C. Streptavidin was immobilised by
20 standard amine coupling methods.

The interactions between the high affinity A6 Tax TCR containing a novel inter-chain bond and the HLA-A2 Tax complex or an irrelevant HLA-A2 NY-ESO combination, the production of which is described above, were analysed on a BIAcore 3000™
25 surface plasmon resonance (SPR) biosensor. SPR measures changes in refractive index expressed in response units (RU) near a sensor surface within a small flow cell, a principle that can be used to detect receptor ligand interactions and to analyse their affinity and kinetic parameters. The probe flow cells were prepared by immobilising the individual HLA-A2 peptide complexes in separate flow cells via binding between
30 the biotin cross linked onto β2m and streptavidin which have been chemically cross linked to the activated surface of the flow cells. The assay was then performed by

passing sTCR over the surfaces of the different flow cells at a constant flow rate, measuring the SPR response in doing so. Initially, the specificity of the interaction was verified by passing soluble A6 TCR at a constant flow rate of 5 $\mu\text{l min}^{-1}$ over four different surfaces; one coated with ~1000 RU of HLA-A2 Tax complex, the second
 5 coated with ~1000 RU of HLA-A2 NY-ESO complex, and two blank flow cells coated only with streptavidin (see Figure 15).

The increased affinity of the mutated soluble A6 TCR made calculation of the k_d for the interaction of this moiety with the HLA-A2 Tax complex difficult. However, the
 10 half-life ($t_{1/2}$) for the interaction was calculated to be 51.6 minutes (see Figure 16), which compares to a $t_{1/2}$ for the wild-type interaction of 7.2 seconds.

Example 11 – Production of vector encoding a soluble NY-ESO TCR containing a novel disulphide bond.

15 The β chain of the soluble A6 TCR prepared in Example 1 contains in the native sequence a BglII restriction site (AAGCTT) suitable for use as a ligation site.

PCR mutagenesis was carried as detailed below to introduce a BamHI restriction site
 20 (GGATCC) into the α chain of soluble A6 TCR, 5' of the novel cysteine codon. The sequence described in Figure 2a was used as a template for this mutagenesis. The following primers were used:

| BamHI |

25 5' -ATATCCAGAACCCgGAtCCTGCCGTGTA-3'
 5' -TACACGGCAGGAaTCcGGGTCTGGATAT-3'

100 ng of plasmid was mixed with 5 μl 10 mM dNTP, 25 μl 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted
 30 to 240 μl with H_2O . 48 μl of this mix was supplemented with primers diluted to give a final concentration of 0.2 μM in 50 μl final reaction volume. After an initial

denaturation step of 30 seconds at 95°C, the reaction mixture was subjected to 15 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs).

5 10 µl of the digested reaction was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K₂HPO₄, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by
10 automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

CDNA encoding NY-ESO TCR was isolated from T cells according to known techniques. CDNA encoding NY-ESO TCR was produced by treatment of the mRNA
15 with reverse transcriptase.

In order to produce a vector encoding a soluble NY-ESO TCR incorporating a novel disulphide bond, A6 TCR plasmids containing the α chain BamHI and β chain BglII restriction sites were used as templates. The following primers were used:

20

| NdeI |

5' -GGAGATATACATATGCAGGAGGTGACACAG-3'

5' -TACACGGCAGGATCCGGGTTCTGGATATT-3'

25

| BamHI |

| NdeI |

5' -GGAGATATACATATGGGTGTCACTCAGACC-3'

5' -CCCAAGCTTAGTCTGCTCTACCCAGGCCTCGGC -3'

| BglII |

30

NY-ESO TCR α and β-chain constructs were obtained by PCR cloning as follows.

PCR reactions were performed using the primers as shown above, and templates containing the native NY-ESO TCR chains. The PCR products were restriction digested with the relevant restriction enzymes, and cloned into pGMT7 to obtain expression plasmids. The sequence of the plasmid inserts were confirmed by automated DNA sequencing. Figures 17a and 17b show the DNA sequence of the mutated NY-ESO TCR α and β chains respectively, and Figures 18a and 18b show the resulting amino acid sequences.

Example 12 - Construction of phage display vectors and cloning of DNA encoding NY-ESO TCR α and β chains into the phagemid vectors.

DNA encoding soluble NY-ESO TCR α and β chains incorporating novel cysteine codons to facilitate the formation of a non-native disulfide inter-chain bond, produced as described in Example 11 were incorporated into the phagemid vector pEX746 as follows.

The DNA encoding the two NY-ESO TCR chains were individually subjected to PCR in order to introduce cloning sites compatible with the pEX746 phagemid vector (containing DNA encoding A6 TCR clone 7) using the following primers:

For the NY-ESO TCR alpha chain

TRAV21

GCCGGCCATGGCCAAACAGGAGGTGACGCAGATTCCT

YOL6

CTTCTTAAAGAATTCTTAATTAACCTAGGTTATTAGGAACTTTCTGGGCTG
GGGAAG

For the NY-ESO TCR beta chain

TRBV6-1/2/3/5/6/7/8/9

TCACAGCGCGCAGGCTGGTGTCACTCAGACCCCAA

RT1
CGAGAGCCCGTAGAACTGGACTTG

The molecular cloning methods for constructing the vectors are described in
5 "Molecular cloning: A laboratory manual, by J. Sambrook and D. W. Russell".
Primers listed in table-1 are used for construction of the vectors. A example of the
PCR programme is 1 cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 5
seconds, 53°C for 5 seconds and 72°C for 90 seconds, followed by 1 cycles of 72°C
for 10 minutes, and then hold at 4°C. The Expand hifidelity Taq DNA polymerase is
10 purchased from Roche.

DNA encoding the clone 7 A6 TCR β chain was removed from pEX746 by digestion
with restrictions enzymes BssHII and BglII. The correspondingly digested PCR DNA
encoding the NY-ESO β chain was then substituted into the phagemid by ligation. The
15 sequence of the cloning product was verified by automated sequencing.

Similarly, DNA encoding the clone 7 A6 TCR α chain was removed from pEX746 by
digestion with restrictions enzymes NcoI and AvrII. The correspondingly digested
PCR DNA encoding the NY-ESO α chain was then substituted into the phagemid
20 already containing DNA encoding the NY-ESO TCR β chain by ligation. The
sequence of the cloning product was verified by automated sequencing.
Figure 19 details the DNA and amino acid sequence of the NY-ESO TCR α and β
chain as well as surrounding relevant sequence incorporated in the phagemid
(pEX746:NY-ESO) respectively. The sequence preceeding the NcoI site (Figure 19) is
25 the same as pEX746.

30

Example 13 – Expression of fusions of bacterial coat protein and heterodimeric NY-ESO TCR in E. coli.

Phage particles displaying the heterodimeric NY-ESO TCR containing a non-native disulfide inter-chain bond were prepared using methods described previously for the generation of phage particles displaying antibody scFvs (Li *et al*, 2000, Journal of Immunological Methods **236**: 133-146) with the following modifications. *E. coli* TG 1 cells containing pEX746:NY-ESO phagemid (i.e. the phagemid encoding the soluble NY-ESO TCR α chain and an NY-ESO TCR β chain fused to the phage gIII protein produced as described in Example 12) were used to inoculate 10 ml of 2x TY (containing 100 μ g/ml of ampicillin and 2% glucose), and then the culture was incubated with shaking at 37°C overnight (16 hours). 50 μ l of the overnight culture was used to inoculate 10 ml of 2x TY (containing 100 μ g/ml of ampicillin and 2% glucose), and then the culture was incubated with shaking at 37°C until OD_{600nm} = 0.8. HYPERPHAGE Helper phage was added to the culture to the final concentration of 5 X 10⁹ pfu/ml. The culture was then incubated at 37°C stationary for thirty minutes and then with shaking at 200 rpm for further 30 minutes. The medium of above culture was then made up to 50 ml with 2x TY (containing 100 μ g/ml of ampicillin and 25 μ g/ml of kanamycin), the culture was then incubated at 25°C with shaking at 250 rpm for 36 to 48 hours. The culture was then centrifuged at 4°C for 30 minutes at 4000 rpm. The supernatant was filtrated through a 0.45 μ m syringe filter and stored at 4°C for further concentration. The supernatant was then concentrated by PEG precipitation and re-suspended in PBS at 10% of the original stored volume.

Example 14 – Detection of functional heterodimeric NY-ESO TCR containing a non-native disulfide inter-chain bond on filamentous phage particles

The presence of functional (HLA-A2-NY-ESO binding) NY-ESO TCR displayed on the phage particles in the concentrated suspension prepared in Example 13 was detected using the phage ELISA methods described in Example 4. Figure 20 shows the specific binding of phage particles displaying the NY-ESO TCR to HLA-A2-NY-ESO in a phage ELISA assay.

Claims:

1. A proteinaceous particle displaying on its surface a T-cell receptor (TCR), characterised in that
- 5
- (i) the proteinaceous particle is a ribosome and the TCR is a single chain TCR (scTCR) polypeptide, or
- (ii) the proteinaceous particle is a phage particle or a cell surface
- 10 protein or polypeptide and the TCR is a human scTCR or a human dimeric T-cell receptor (dTCR) polypeptide pair, or
- (iii) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a non-human dTCR polypeptide pair, or
- 15
- (iv) the proteinaceous particle is a phage particle or a cell surface protein or polypeptide and the TCR is a scTCR polypeptide comprising TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the
- 20 latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain, and a disulfide bond which has no equivalent in native T cell receptors links residues of the constant region sequences.
- 25
2. A proteinaceous particle, displaying on its surface a dimeric T-cell receptor (dTCR) polypeptide pair, or a single chain T-cell receptor (scTCR) polypeptide wherein
- 30 the dTCR polypeptide pair is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains,

and the scTCR is constituted by TCR amino acid sequences corresponding to extracellular constant and variable region sequences present in native TCR chains and a linker sequence, the latter linking a variable region sequence corresponding to that of one chain of a native TCR to a constant region sequence corresponding to a constant region sequence of another native TCR chain;

the variable region sequences of the dTCR polypeptide pair or scTCR polypeptide are mutually orientated substantially as in native TCRs; and

in the case of the scTCR polypeptide a disulfide bond which has no equivalent in native T cell receptors links residues of the polypeptide.

3. A proteinaceous particle as claimed in claim 1 or claim 2, which is a phage particle.

4. A proteinaceous particle as claimed in claim 1 or claim 2, which is a cell surface protein or polypeptide.

5. A proteinaceous particle as claimed in claim 1 or claim 2, which is a ribosome.

6. A proteinaceous particle as claimed in any of the preceding claims wherein the C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, is linked by a peptide bond to a surface exposed residue of the proteinaceous particle.

7. A proteinaceous particle as claimed in any of claims 1 to 4 wherein the C-terminus of one member of the dTCR polypeptide pair, or the C-terminus of the scTCR polypeptide, is linked by a disulfide bond to a surface exposed cysteine residue of the proteinaceous particle.

8. A proteinaceous particle as claimed in any of the preceding claims wherein an scTCR polypeptide is displayed which comprises

5 a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region

a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus of an amino acid sequence
10 corresponding to a TCR β chain constant region extracellular sequence, and

a linker sequence linking the C terminus of the first segment to the N terminus of the second segment.

15 9. A proteinaceous particle as claimed in any of claims 1 to 7 wherein an scTCR polypeptide is displayed which comprises

a first segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region
20

a second segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence, and

25 a linker sequence linking the C terminus of the first segment to the N terminus of the second segment.

10. A proteinaceous particle as claimed in any of claims 1 to 7 wherein a scTCR polypeptide is displayed which has
30

a first segment constituted by an amino acid sequence corresponding to a TCR α or δ chain variable region sequence fused to the N terminus of an amino acid sequence corresponding to a TCR α chain constant region extracellular sequence,

5 a second segment constituted by an amino acid sequence corresponding to a TCR β or γ chain variable region fused to the N terminus of an amino acid sequence corresponding to TCR β chain constant region extracellular sequence,

10 a linker sequence linking the C terminus of the first segment to the N terminus of the second segment, or vice versa, and

a disulfide bond between the first and second chains, said disulfide bond being one which has no equivalent in native $\alpha\beta$ or $\gamma\delta$ T cell receptors,

15 the length of the linker sequence and the position of the disulfide bond being such that the variable region sequences of the first and second segments are mutually orientated substantially as in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

20 11. A proteinaceous particle as claimed in claim 10 wherein the linker sequence has the formula -P-AA-P- wherein P is proline and AA represents an amino acid sequence wherein the amino acids are glycine and serine.

25 12. A proteinaceous particle as claimed in claim 10 or claim 11 wherein the linker sequence links the C terminus of the first segment to the N terminus of the second segment.

13. A proteinaceous particle as claimed in claim 12 wherein the linker sequence consists of from 26 to 41 amino acids.

30 14. A proteinaceous particle as claimed in claim 13 wherein the linker sequence consists of 29, 30, 31 or 32 amino acids.

15. A proteinaceous particle as claimed in claim 13 wherein the linker sequence consists of 33, 34, 35 or 36 amino acids.

5 16. A proteinaceous particle as claimed in claim 13 wherein the linker sequence has the formula -PGGG-(SGGGG)₅-P- wherein P is proline, G is glycine and S is serine.

10 17. A proteinaceous particle as claimed in claim 13 wherein the linker sequence has the formula -PGGG-(SGGGG)₆-P- wherein P is proline, G is glycine and S is serine.

15 18. A proteinaceous particle as claimed in any of claims 1 to 4, 6 or 7 wherein a dTCR polypeptide pair is displayed which is constituted by
a first polypeptide wherein a sequence corresponding to a TCR α or δ chain variable region sequence is fused to the N terminus of a sequence corresponding to a TCR α chain constant region extracellular sequence, and

20 a second polypeptide wherein a sequence corresponding to a TCR β or γ chain variable region sequence fused to the N terminus a sequence corresponding to a TCR β chain constant region extracellular sequence,

25 the first and second polypeptides being linked by a disulfide bond which has no equivalent in native $\alpha\beta$ or $\gamma\delta$ T cell receptors.

30 19. A proteinaceous particle as claimed in any preceding claim wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to $\alpha\beta$ TCR extracellular constant and variable region sequences.

20. A proteinaceous particle as claimed in any of claims 1 to 18 wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to extracellular $\alpha\beta$ TCR constant region sequences and $\gamma\delta$ TCR variable region sequences.

5

21. A proteinaceous particle as claimed in any preceding claim wherein the displayed dTCR polypeptide pair or scTCR polypeptide have amino acid sequences corresponding to non-human extracellular $\alpha\beta$ TCR constant region sequences and human TCR variable region sequences.

10

22. A proteinaceous particle as claimed in any of the preceding claims wherein an amino acid sequence of one member of the displayed dTCR polypeptide pair, or an amino acid sequence of the displayed scTCR, corresponds to a native TCR extracellular constant chain Ig domain sequence.

15

23. A proteinaceous particle as claimed in any of the preceding claims wherein the displayed dTCR polypeptide pair or displayed scTCR, includes sequences corresponding to native TCR extracellular constant chain Ig domain sequences.

20

24. A proteinaceous particle as claimed in claim 23 wherein a disulfide bond links amino acid residues of the said constant chain Ig domain sequences, which disulfide bond has no equivalent in native TCRs.

25

25. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues corresponding to amino acid residues whose β carbon atoms are less than 0.6 nm apart in native TCRs.

30

26. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 48 of exon 1 of TRAC*01 and Ser 57 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.

27. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 45 of exon 1 of TRAC*01 and Ser 77 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.
- 5 28. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Tyr 10 of exon 1 of TRAC*01 and Ser 17 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.
- 10 29. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Thr 45 of exon 1 of TRAC*01 and Asp 59 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.
- 15 30. A proteinaceous particle as claimed in claim 24 wherein the said disulfide bond is between cysteine residues substituted for Ser 15 of exon 1 of TRAC*01 and Glu 15 of exon 1 of TRBC1*01 or TRBC2*01 or the non-human equivalent thereof.
- 20 31. A proteinaceous particle as claimed in any of claims 23 to 30 wherein the sequences corresponding to native TCR extracellular constant chain Ig domain sequences are truncated at their C-termini relative to said native sequences such that the cysteine residues which form the native interchain disulphide bond are excluded.
- 25 32. A proteinaceous particle as claimed in any of claims 23 to 30 wherein in the sequences corresponding to native TCR extracellular constant chain Ig domain sequences the cysteine residues which form the native interchain disulphide bond are substituted by non-cysteine residues.
33. A proteinaceous particle as claimed in claim 32 wherein the cysteine residues which form the native interchain disulfide bond are substituted by serine or alanine.

34. A proteinaceous particle as claimed in any of the preceding claims wherein in the displayed dTCR or scTCR there is no unpaired cysteine residue corresponding an unpaired cysteine residue present in a native TCR.

5 35. A proteinaceous particle as claimed in any of claims 23 to 34 wherein the sequences corresponding to native TCR extracellular constant chain Ig domain sequences are truncated N-terminal to residues corresponding to those which form the non-native interchain disulphide bond.

10 36. A diverse library of dTCR polypeptide pairs or scTCR polypeptides displayed on proteinaceous particles said dTCR polypeptide pairs or scTCR polypeptides having the structural features defined in any of claims 8 to 35.

15 37. A diverse library as claimed in claim 36 wherein the diversity resides in the variable region(s) of the dTCR or scTCR polypeptides.

38. A diverse library as claimed in claim 36 wherein the diversity resides in one or more of the complementarity determining regions of the variable region(s) of the dTCR or scTCR polypeptides.

20 39. A diverse library as claimed in any of claims 36 to 38 wherein the dTCR or scTCR polypeptides are displayed on phage particles.

25 40. Nucleic acid encoding (a) one chain of a dTCR polypeptide pair and (b) the other chain of a dTCR polypeptide pair fused to a nucleic acid sequence encoding a particle forming protein, or nucleic acid encoding a scTCR polypeptide fused to a nucleic acid sequence encoding a particle forming protein, the dTCR pair or scTCR having the structural features defined in any of claims 8 to 35.

30 41. An expression vector comprising nucleic acid as claimed in claim 40.

42. An expression vector as claimed in claim 41 which is a phagemid or phage genome vector.

5 43. A phagemid or phage genome vector as claimed in claim 42 wherein the nucleic acid sequence(s) which encode the particle-forming protein is (are) derived from a filamentous phage.

10 44. A phagemid or phage genome vector as claimed in claim 43 wherein the said nucleic acid sequence(s) encode(s) bacteriophage gIII or gVIII coat proteins.

45. An expression vector as claimed in any of claims 41 to 44 containing a sequence or sequences which limit constitutively or inducibly the amount of TCR polypeptide expressed by the vector to a desired level.

15 46. An expression vector as claimed in claim 45 wherein the said sequence(s) is (are) weak promoter sequence(s)

20 47. An expression vector as claimed in claim 45 wherein the said sequence(s) is (are) mutated ribosome binding site(s).

48. An expression vector as claimed in claim 45 wherein the said sequence(s) is (are) miss-sense suppressor stop codon(s).

25 49. An expression vector as claimed in claim 45 wherein the said sequence(s) is (are) mutated start codon(s).

30 50. An expression vector as claimed in claim 45 wherein the said sequence(s) contain(s) a number of codons less preferred by the expression system being utilised.

51. A host cell comprising nucleic acid as claimed in claim 40, or an expression vector as claimed in any of claims 41 to 50.

52. A host cell harbouring a phagemid expression vector as claimed in any of claims 42 to 50, and a helper phage.

53. A method for the identification of TCRs with a specific characteristic, said method comprising subjecting a diverse library of TCRs displayed on proteinaceous particles as claimed in any of claims 36 to 39 to an assay which measures said characteristic, identifying those proteinaceous particles which display a TCR with the desired characteristic and isolating these proteinaceous particles.

54. A method as claimed in claim 53 wherein the specific characteristic is increased affinity for a TCR ligand.

15

55. A method for detecting TCR ligand complexes, which comprises:

- (i) providing a TCR-displaying proteinaceous particle as claimed in any of claims 1 to 39;
- (ii) contacting said TCR-displaying proteinaceous particle with a putative ligand complex; and
- (iii) detecting binding of the said TCR-displaying proteinaceous particle to the putative ligand complexes.

20

56. A method as claimed in claim 55 wherein the putative TCR ligand complexes are peptide-MHC complexes.

25

57. A method of identifying an inhibitor of the interaction between a TCR-displaying proteinaceous particle as claimed in any one of claims 1 to 39, and a TCR-binding ligand comprising contacting the TCR-displaying proteinaceous particle with a TCR-binding ligand, in the presence of and in the absence of a test compound, and determining whether the presence of the test compound reduces binding of the TCR-

30

displaying proteinaceous particle to the TCR-binding ligand, such reduction being taken as identifying an inhibitor.

58. A TCR specific for a given TCR ligand, which (i) has the structural features defined in any of claims 8 to 35, (ii) is mutated in the variable region(s) relative to the native TCR specific for said TCR ligand and which (iii) has a K_d for the said TCR ligand less than that of the native TCR.

59. A TCR specific for a given TCR ligand, which (i) has the structural features defined in any of claims 8 to 35, (ii) is mutated in the variable region(s) relative to the native TCR specific for said TCR ligand and which (iii) has a K_d for the said TCR ligand less than that of the native TCR as measured by Surface Plasmon Resonance.

60. A TCR specific for a given TCR ligand, which (i) has the structural features defined in any of claims 8 to 35, (ii) is mutated in the variable region(s) relative to the native TCR specific for said TCR ligand and which (iii) has an off-rate (k_{off}) for the said TCR ligand less than that of the native TCR

61. A TCR specific for a given TCR ligand, which (i) has the structural features defined in any of claims 8 to 35, (ii) is mutated in the variable region(s) relative to the native TCR specific for said TCR ligand and which (iii) has an off-rate (k_{off}) for the said TCR ligand less than that of the native TCR as measured by Surface Plasmon Resonance.

62. A dimeric TCR as claimed in any of claims 58 to 61 which has the structural features of an $\alpha\beta$ heterodimeric TCR defined in any of claims 18 to 35.

63. A TCR as claimed in any of claims 58 to 62 which is mutated relative to the native TCR in at least one complementarity determining region.

64. A TCR as claimed in any of claims 58 to 63 which is specific for a given MHC type or types.
- 5 65. A TCR as claimed in any of claims 58 to 63 which is specific for a given pMHC.
66. A TCR as claimed in any of claims 58 to 63 or 65 which is specific for the HLA-A2 Tax peptide (LLFGYPVYV) complex.
- 10 67. A TCR as claimed in any of claims 58 to 66 associated with a therapeutic or imaging compound.
68. A TCR as claimed in any of claims 58 to 66 associated with a cytotoxic compound.
- 15 69. A TCR as claimed in claim 68 wherein the TCR is specific for the HLA-A2 Tax peptide (LLFGYPVYV) complex.
70. A method of treatment of HTLV-1 infection comprising administering to a
20 subject suffering such infection an effective amount of a TCR as claimed in claim 66 or claim 68.
71. The use of a TCR as claimed in claim 66 or claim 68 in the preparation of a
25 composition for the treatment of HTLV-1 infection.
-

Figure 1a

atgcagaaggaagtggagcagaactctggacccctcagtgttccagagggagccatt
gcctctctcaactgcacttacagtgaccgaggttcccagtccttcttctggtacaga
caatattctgggaaaagccctgagttgataatgtccatatactccaatggtgacaaa
gaagatggaagggtttacagcacagctcaataaagccagccagtatgttttctctgctc
atcagagactcccagcccagtgattcagccacctacctctgtgccgttacaactgac
agctgggggaaattgcagtttggagcagggacccaggttggtggtcaccccagatatc
cagaaccctgaccctgccgtgtaccagctgagagactctaaatccagtgacaagtct
gtctgcctattcaccgattttgattctcaaacaatgtgtcacaagtaaggattct
gatgtgtatatcacagacaaatgtgtgctagacatgaggtctatggacttcaagagc
aacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaac
aacagcattattccagaagacaccttcttccccagcccagaaagttcctaa

Figure 1b

atgaacgctggtgtcactcagacccccaaaattccaggtcctgaagacaggacagagc
atgacactgcagtgtgccaggatatgaaccatgaatacatgtcctgggtatcgacaa
gaccagggcatggggctgaggctgattcattactcagttggtgctggtatcactgac
caaggagaagtccccaatgggtacaatgttctccagatcaaccacagaggatttccg
ctcaggctgctgtcggctgctccctcccagacatctgtgtacttctgtgccagcagg
ccgggactagcgggagggcgaccagagcagtagtctcgggcccgggcaccaggctcacg
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gaagcagagatctcccacacccaaaaggccacactggtgtgcctggccacaggcttc
taccgccaccacgtggagctgagctggtgggtgaatgggaaggaggtgcacagtggg
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gctctgagcagccgcctgagggtctcggccaccttctggcaggacccccgcaaccac
ttccgctgtcaagtccagttctacgggctctcggagaatgacgagtggaaccaggat
agggccaaacccgctcacccagatcgtcagcgcggaggcctggggtagagcagactaa

Figure 2a

MQ
K₁EVEQNSGPL SVPEGAIASL NCTYSDRGSQ SFFWYRQYSG KSPELIMSIY
SNGDKEDGRF TAQLNKASQY VSLLIRDSQP SDSATYLCAY TTDSWGKLQF
GAGTQVVVTP DIQNPDPVAVY QLRDSKSSDK SVCLFTDFDS QTNVSQSKDS
DVYITDKCVL DMRSMDFKSN SAVAWSNKSD FACANAFNNS IIPEDTFFPS
PESS*

Figure 2b

M
N₁AGVTQTPKF QVLKTGQSMT LQCAQDMNHE YMSWYRQDPG MGLRLIHYSV
GAGITDQGEV PNGYNVSRST TEDFPLRLLS AAPSQTSVYF CASRPGLAGG
RPEQYFGPGT RLTVTEDLKN VFPPEVAVFE PSEAEISHTQ KATLVCLATG
FYPDHVELSW WVNGKEVHSG VCTDPQPLKE QPALNDSRYA LSSRLRVSAT
FWQDPRNHFR CQVQFYGLSE NDEWTQDRAK PVTQIVSAEA WGRAD*

Figure 3

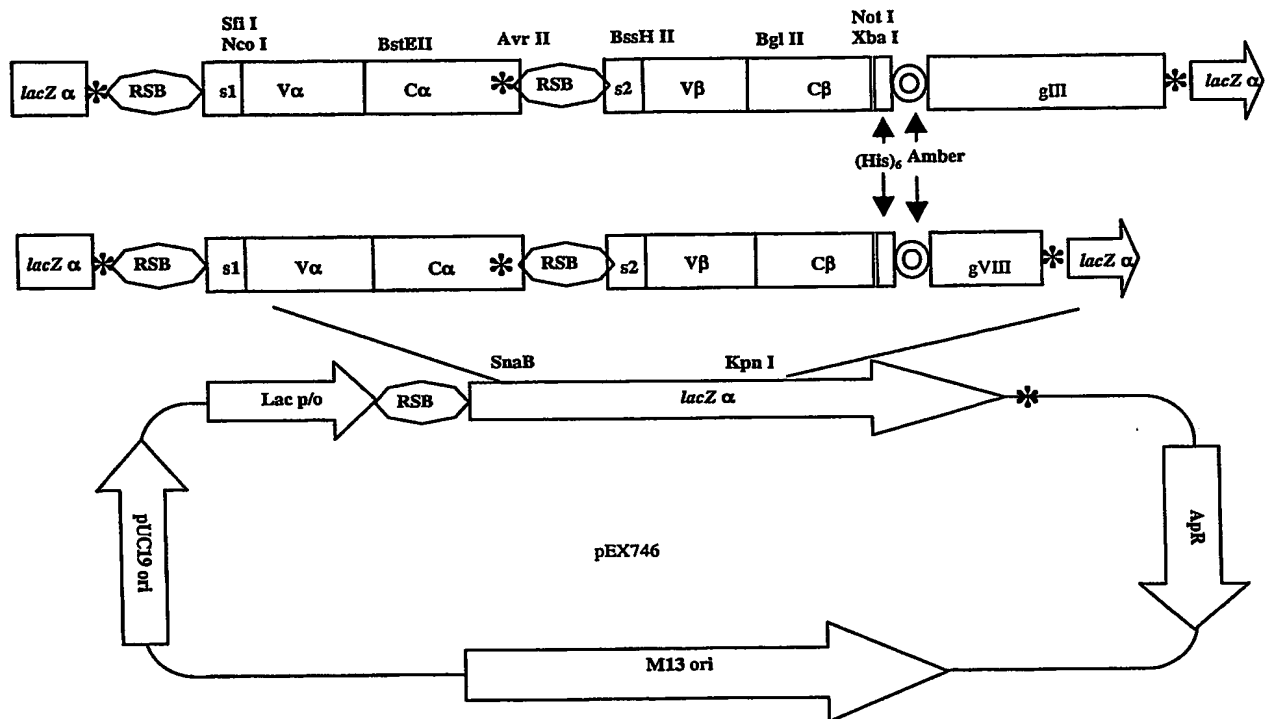


Figure 4

```

1  gttaactacg tcaggtggca cttttcgggg aaatgtggcg ggaaccccta tttgtttatt
61  tttctaaata cattcaaata tgtatccgct catgagacaa taaccctgat aaatgcttca
121 ataataattga aaaaggaaga gtatgagtat tcaacatttc cgtgtcgccc ttattccctt
181 ttttgcggca ttttgccttc ctgttttttg tcacccagaa acgctgggtg aagtaaaaga
241 tgctgaagat cagttgggtg cacgagtggg ttacatcgaa ctggatctca acagcggtaa
301 gatccttgag agttttcgcc ccgaagaacg ttctccaatg atgagcactt ttaaagttct
361 gctatgtggc gcggtattat cccgtgttga cgccgggcaa gagcaactcg gtcgccgcat
421 acactattct cagaatgact tggttgagta ctcaccagtc acagaaaagc atcttacgga
481 tggcatgaca gtaagagaat tatgcagtgc tgccataacc atgagtgata acactgcggc
541 caacttactt ctgacaacga tcggaggacc gaaggagcta accgcttttt tgcaacaacat
601 gggggatcat gtaactcgcc ttgatcgttg ggaaccggag ctgaatgaag ccataccaaa
661 cgacgagcgt gacaccacga tgccctgtagc aatggcaaca acgttgcgca aactattaac
721 tggcgaacta cttactctag cttcccggca acaattaata gactggatgg aggcggataa
781 agttgcagga ccacttctgc gctcgccctt tccggctggc tggttttatt ctgataaatc
841 tggagccggt gagcgtgggt ctgcggttat cattgcagca ctggggccag atggttaagcc
901 ctcccgtatc gtagtttatct acacgacggg gagtcaggca actatggatg aacgaaatag
961 acagatcgct gagataggtg cctcactgat taagcattgg taactgtcag accaagttta
1021 ctcatatata ctttagattg atttaccctg gttgataatc agaaaagccc caaaaacagg
1081 aagattgtat aagcaaatat ttaaattgta aacgttaata ttttgttaa attcgcgtta
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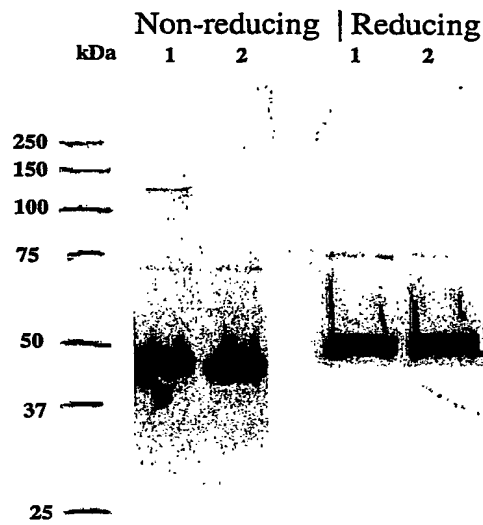
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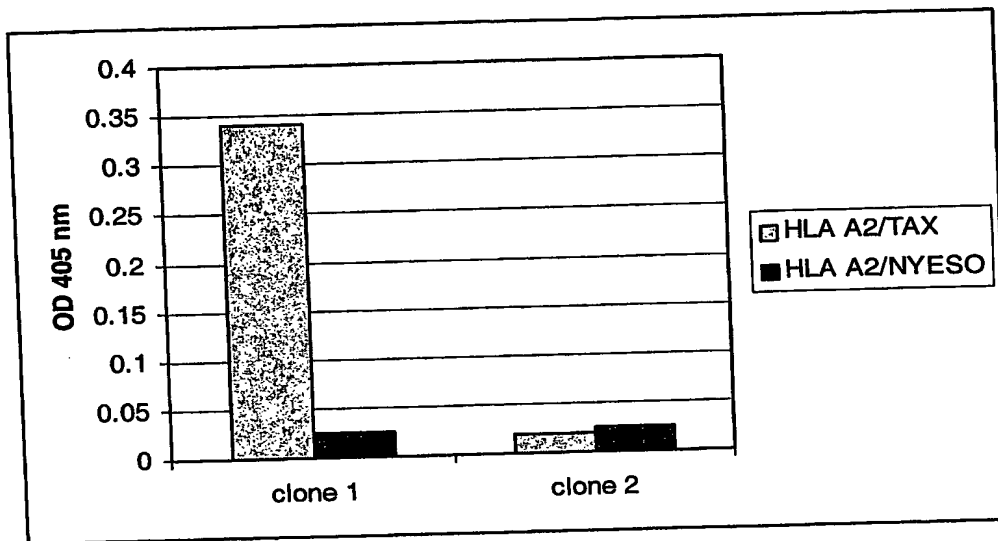
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Figure 5



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Figure 6



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Figure 7a

Schematic diagram of the A6 scTCR-C-Kappa ribosome display construct

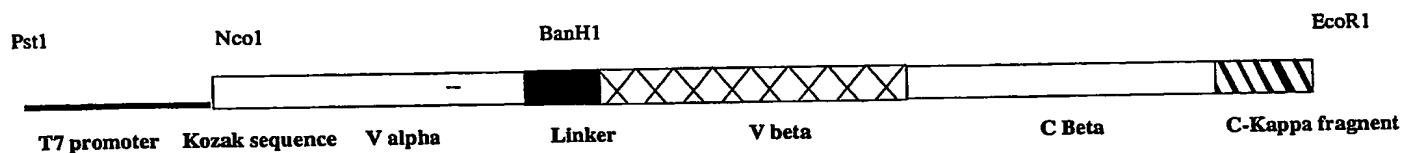


Figure 7b

	T7 Promoter sequence	Kozak sequence
	PstI	NcoI
	-----	-----
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	TCGACGTCGA TTATGCTGAG TGATATCCTT GTCCGGTGGT ACCCGGTCTT	
	. E V E Q N S G P L S V P E G A I A .	
1251	GGAAGTGGAG CAGAACTCTG GACCCCTCAG TGTTCAGAG GGAGCCATTG	
	CCTTCACCTC GTCTTGAGAC CTGGGGAGTC ACAAGGTCTC CCTCGGTAAC	
	. S L N C T Y S D R G S Q S F F W	
1251	CCTCTCTCAA CTGCACTTAC AGTGACCGAG GTTCCAGTC CTTCTTCTGG	
	GGAGAGAGTT GACGTGAATG TCACTGGCTC CAAGGGTCAG GAAGAAGACC	
	. Y R Q Y S G K S P E L I M S I Y S .	
1251	TACAGACAAT ATTCTGGGAA AAGCCCTGAG TTGATAATGT CCATATACTC	
	ATGTCTGTTA TAAGACCCTT TTCGGGACTC AACTATTACA GGTATATGAG	
	. N G D K E D G R F T A Q L N K A S .	
1251	CAATGGTGAC AAAGAAGATG GAAGGTTTAC AGCACAGCTC AATAAAGCCA	
	GTTACCACTG TTTCTTCTAC CTTCCAAATG TCGTGTGCGAG TTATTTTCGGT	
	. Q Y V S L L I R D S Q P S D S A	
1251	GCCAGTATGT TTCTCTGCTC ATCAGAGACT CCCAGCCCAG TGATTCAGCC	
	CGGTCATACA AAGAGACGAG TAGTCTCTGA GGGTCGGGTC ACTAAGTCGG	
	PvuII	

	T Y L C A V T T D S W G K L Q F G .	
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	TGGATGGAGA CACGGCAATG TTGACTGTGCG ACCCCCTTTA ACGTCAAACC	
	AgeI	BamHI
	-----	-----
	. A G T Q V V V T G G G G S G G G G .	
1251	AGCAGGGACC CAGGTTGTGG TCACCGGTGG AGGCGGTTCA GGCGGAGGTG	

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TCGTCCCTGG GTCCAACACC AGTGGCCACC TCCGCCAAGT CCGCCTCCAC
BamHI
-----
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-----
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CTAGGCCGCC ACCGCCAGC TTGCGACCAC AGTGAGTCTG GGGTTTAAAG
PstI
-----
Q V L K T G Q S M T L Q C A Q D M .
-----
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GTCCAGGACT TCTGTCCTGT CTCGTACTGT GACGTCACAC GGGTCCTATA
. N H E Y M S W Y R Q D P G M G L R .
-----
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CTTGGTACTT ATGTACAGGA CCATAGCTGT TCTGGGTCCG TACCCCGACT
. L I H Y S V G A G I T D Q G E V
-----
1251 GGCTGATTCA TTA CTCACTGTT GGTGCTGGTA TCACTGACCA AGGAGAACTC
CCGACTAAGT AATGAGTCAA CCACGACCAT AGTGACTGGT TCCTCTTCAG
P N G Y N V S R S T T E D F P L R .
-----
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GGGTACCGA TGTTACAGAG GTCTAGTTGG TGTCTCTTAA AGGCGGAGTC
. L L S A A P S Q T S V Y F C A S R .
-----
1251 GCTGCTGTCG GCTGCTCCCT CCCAGACATC TGTGTACTTC TGTGCCAGCA
CGACGACAGC CGACGAGGGA GGGTCTGTAG ACACATGAAG ACACGGTCGT
ScaI
-----
. P G L A G G R P E Q Y F G P G T
-----
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R L T V T E D L K N V F P P E V A .
-----
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BglII
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-----
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. V C L A T G F Y P D H V E L S W
-----
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ApaLI
-----
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-----
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. L K E Q P A L N D S R Y A L S S R .
-----
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Bsu36I
-----
. L R V S A T F W Q D P R N H F R
-----
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C Q V Q F Y G L S E N D E W T Q D .

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                        StuI
                        -----
      . R A K P V T Q I V S A E A W G R A .
      -----
1251  TAGGGCCAAA CCCGTCACCC AGATCGTCAG CGCCGAGGCC TGGGGTAGAG
      ATCCCGGTTT GGGCAGTGGG TCTAGCAGTC GCGGCTCCGG ACCCCATCTC
      . D G G G G S L S S T L T L S K A
      -----
1251  CAGACGGTGG AGGCGGTTCA CTCAGCAGCA CCCTGACGCT GAGCAAAGCA
      GTCTGCCACC TCCGCCAAGT GAGTCGTCGT GGGACTGCGA CTCGTTTCGT
      D Y E K H K V Y A C E V T H Q G L .
      -----
1251  GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT
      CTGATGCTCT TTGTGTTTCA GATGCGGACG CTTCAAGTGGG TAGTCCCGGA
                        EcoRI
                        -----
      . S S P V T K S . F N R G E S
      -----
1251  GAGTTCGCCC GTCACAAAGA GCTTCAACCG CGGAGAGTCA TAAGAATTCT
      CTCAAGCGGG CAGTGTCTCT CGAAGTTGGC GCCTCTCAGT ATTCTTAAGA
1251  CAG
      GTC
```

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Figure 8

pUC19-T7 sequence

```

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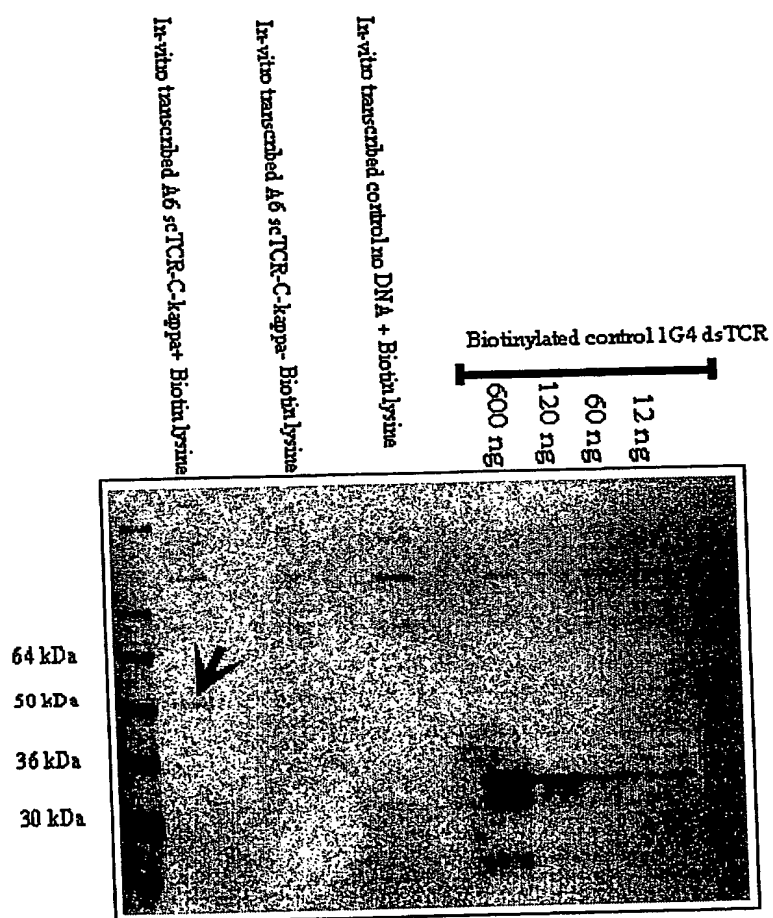
Figure 9

A6 scTCR-C-kappa cloned into pUC19-T7

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451 gtgcccagga tatgaaccat gaatacatgt cctgggtatcg acaagaccca
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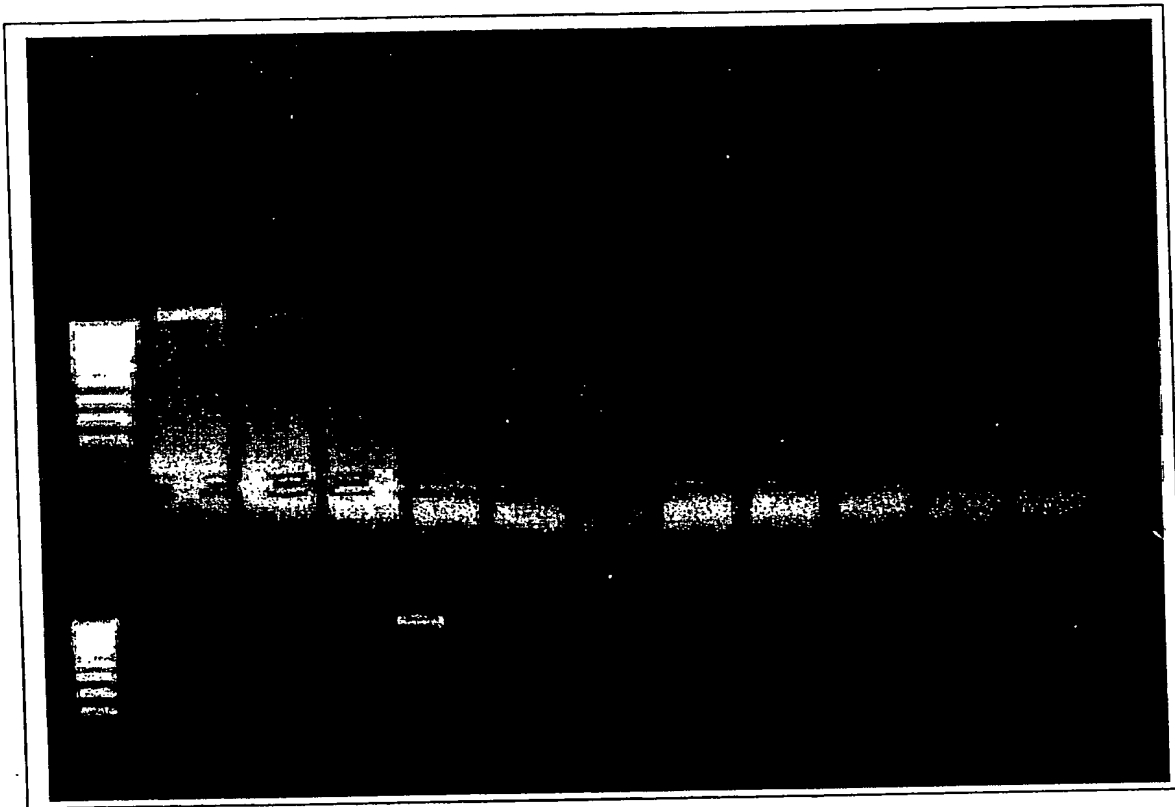
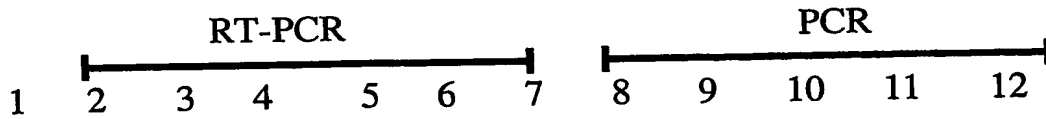
Figure 10



The A6 scTCR-C-Kappa protein is shown in the above western blot with an arrow.

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Figure 11



13 14

15

Lane 1 Bioline 100bp DNA marker

Lane 2 A6scTCR-C-Kappa reaction selected against HLA-A2 TAX beads

Lane 3 A6scTCR-C-Kappa reaction selected against HLA-A2 TAX beads in the presence of 10 microgrammes of soluble A6scTCR

Lane 4 A6scTCR-C-Kappa reaction selected against control beads

Lane 5 Control no DNA reaction selected against HLA-A2-TAX beads

Lane 6 Control no DNA reaction selected against HLA-A2 TAX beads in the presence of 10 microgrammes of soluble A6scTCR

Lane 7 Control no DNA reaction selected against control beads

Lanes 8-12 and lane 13 are as lanes 2-7 except no reverse transcriptase was added just Roche high fidelity taq. These are the DNA contamination controls.

Lane 13 RT-PCR positive control.

Figure 12a (Clone 9)**Clone 9 Mutated A6 TCR β chain DNA sequence**

gctggtgtcactcagacccccaaattccaggtcctgaagacaggacagagcatgacactgcagtggtgccaggatatgaaccat
 gaatacatgtcctggtatcgacaagaccagggcatggggctgaggctgattcattactcagttggtgctggtatcactgaccaagga
 gaagtcccaatggctacaatgtctccagatcaaccacagaggattcccgctcaggctgctgtcggctgctccctcccagacatct
 gtgtactctgtgccagcaggccgggactagcgggaggggtgaccagagcagtagctcgggcccgggcaccaggctcacgggtcac
 agaggacctgaaaaacgtgttcccacccgaggtcgtgtgtttgagccatcagaagcagagatctcccacacccaaaaggcca
 cactggtgtgcttgccacaggcttctaccccgaccacgtggagctgagctggtgggtgaatgggaaggaggtgcacagtgggg
 tctgcacagacccgcagccctcaaggagcagcccgccctcaatgactccagatacgtctgagcagccgcctgaggggtctcgg
 ccaccttctggcaggacccccgcaaccacttccgctgtcaagtccagttctacgggctctcggagaatgacgagtggaaccagga
 tagggccaaacccgtcaccagatcgtcagcgcggaggcctggggtagagcagac

Figure 12b (Clone 9)**Clone 9 Mutated A6 TCR β chain amino acid sequence**

Agvtqtpkfvlktgqsmtlqcaqdmnheymswyrqdpmgrlrlhysvgagitdqgevpngynvsrsttdfplrlsaapsqts
 vyfcasrpglagg*peqyfgpgtrltvtedlknvfppevavfepseaeishtqkatlvclatgfypdhvelswwwngkevhsgvctd
 ppplkeqpalndsryalssrlrvsatfwqprnhfrcqvqfyglsendewtqdrakpvtqivsaeawgrad

* - Denotes the position of the amino acid corresponding to the introduced 'opal' stop codon,
 this will generally result in the substitution of a tryptophan (w) residue into the TCR β chain at
 this point.

Figure 13 (Clone 49)**Clone 49 Mutated A6 TCR β chain DNA sequence**

gctggtgtcactcagaccccaaatccaggtcctgaagacaggacagagcatgacactgtagtgtgccaggatatgaacct
gaatacatgtcctggtatcgacaagacccagggcatggggctgaggctgattcattactcagttggtgctggtatcactgaccaagga
gaagtcccaatggctacaatgtctccagatcaaccacagaggatttcccgctcaggctgctgtcggctgctccctccagacatct
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agaggacctgaaaaacgtgttcccacccgaggtcgtgtgttgagccatcagaagcagagatctccacacccaaaaggcca
cactggtgtgcttgccacaggcttctaccccgaccacgtggagctgagctggtgggtgaatgggaaggagggtgcacagtggg
tctgcacagacccgcagccctcaaggagcagccgcccctcaatgactccagatacgtctgagcagccgctgagggtctcgg
ccaccttctggcaggaccccgcaaccacttccgctgtcaagtccagttctacgggctctcggagaatgacgagtggaaccagga
tagggccaaacccgtcaccagatcgtcagcgccgaggcctggggtagagcagac

Figure 14a (Clone 134)

Clone 134 Mutated A6 TCR β chain DNA sequence

gctggtgtcactcagacccccaaattccaggtcctgaagacaggacagagcatgacactgcagtggtgcccaggatatgaacat
 gaatacatgtcctggtatcgacaagaccagggcatgggctgaggctgattcattactcagttggtgctggtatcactgaccaagga
 gaagtcccaatggctacaatgtctccagatcaaccacagaggattcccgctcaggctgctgtcggctgtccctcccagacatct
 gtgtacttctgtcctcgaggccggggctgatgagtgcggaaccagagcagtactcgggcccgggcaccaggctcacgggtcac
 agaggacctgaaaaacgtgtccacccgaggtcgtgtgttgagccatcagaagcagagatctccacacccaaaaggcca
 cactggtgtgctgcccaccgggttttaccacccgaccacgtggagctgagctgggtgggtgaatgggaaggaggtgcacagtgggt
 ctgcacagacccgcagccctcaaggagcagcccgccctcaatgactccagatacgctctgagcagccgcctgaggggtctcgg
 ccaccttctggcaggacccccgaaccacttccgctgtcaagtcagttctacgggctctcggagaatgacgagtggaaccagga
 tagggccaaaccgctacccagatcgtcagcgccgaggcctggggtagagcagactaagctgaattc

Figure 14b (Clone 134)

Clone 134A Mutated A6 TCR β chain amino acid sequence (BIAcore)

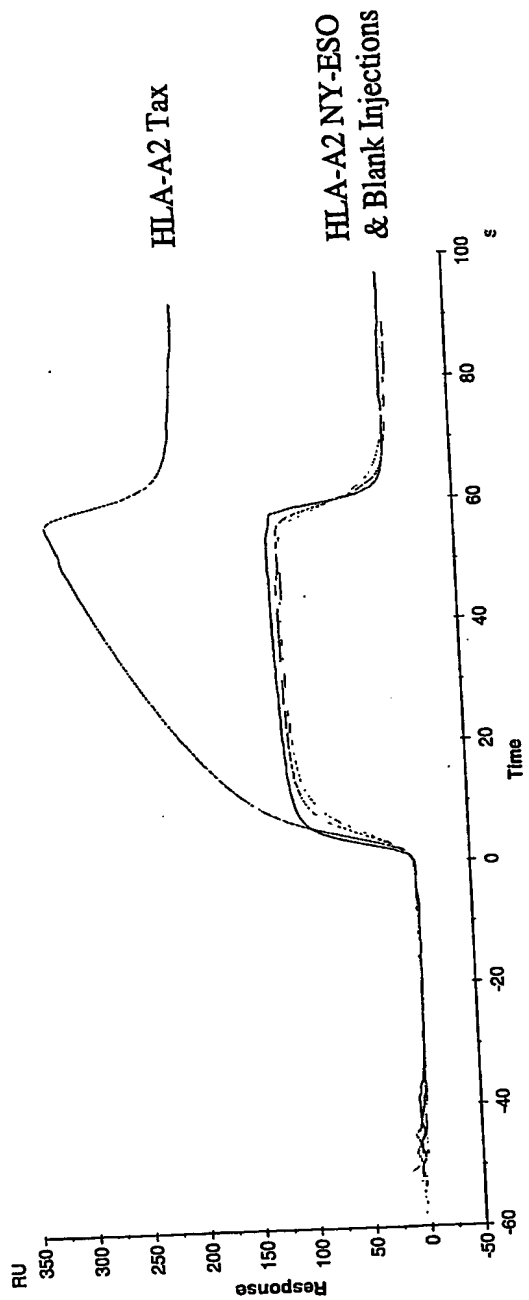
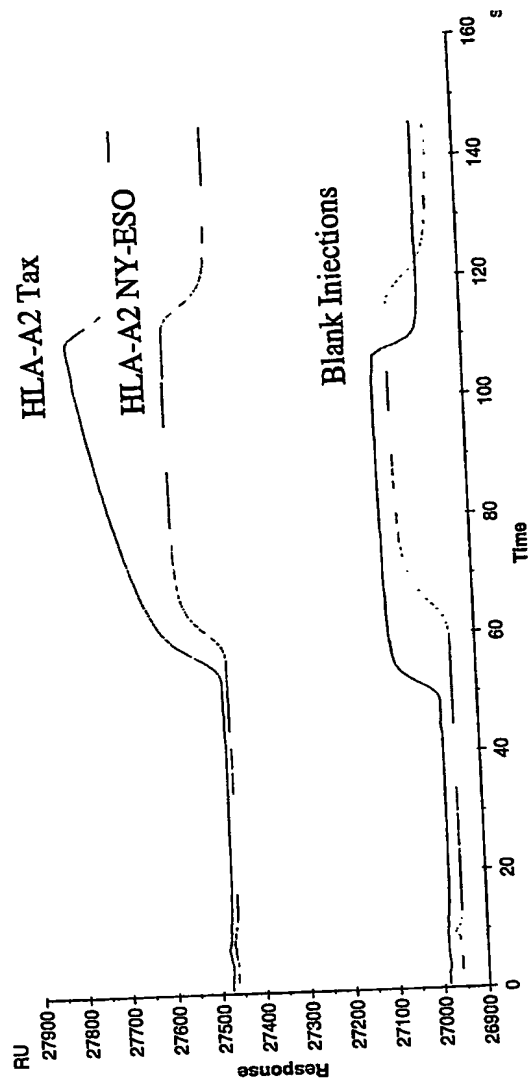
mnagvtqpkfqvltgqsmllqcaqdmnheymswyrqdpmgllrlhysvgagitdqqevpnpngynvsrstedfplrlsaaps
 qtsvyfcasrpglmsaepeqyfgpgtrltvtedlknvfppevavfepseaeishtqkatlvclatgfypdhvelswwwngkevhs
 vctdpqlkeqpalsrlyalssrlvsatfwqdpnrhrcqvfyglsendewtqdrakpvtqivsaeawgrad*

Figure 14c (Clone 134)

Clone 134 Mutated A6 TCR β chain amino acid sequence (ELISA)

agvtqtpkfqvltgqsmllqcaqdmnheymswyrqdpmgllrlhysvgagitdqqevpnpngynvsrstedfplrlsaapsqts
 vyfcasrpglmsaepeqyfgpgtrltvtedlknvfppevavfepseaeishtqkatlvclatgfypdhvelswwwngkevhs
 dpqlkeqpalsrlyalssrlvsatfwqdpnrhrcqvfyglsendewtqdrakpvtqivsaeawgrad

A6 TCR clone 134



19/23

Figure 16

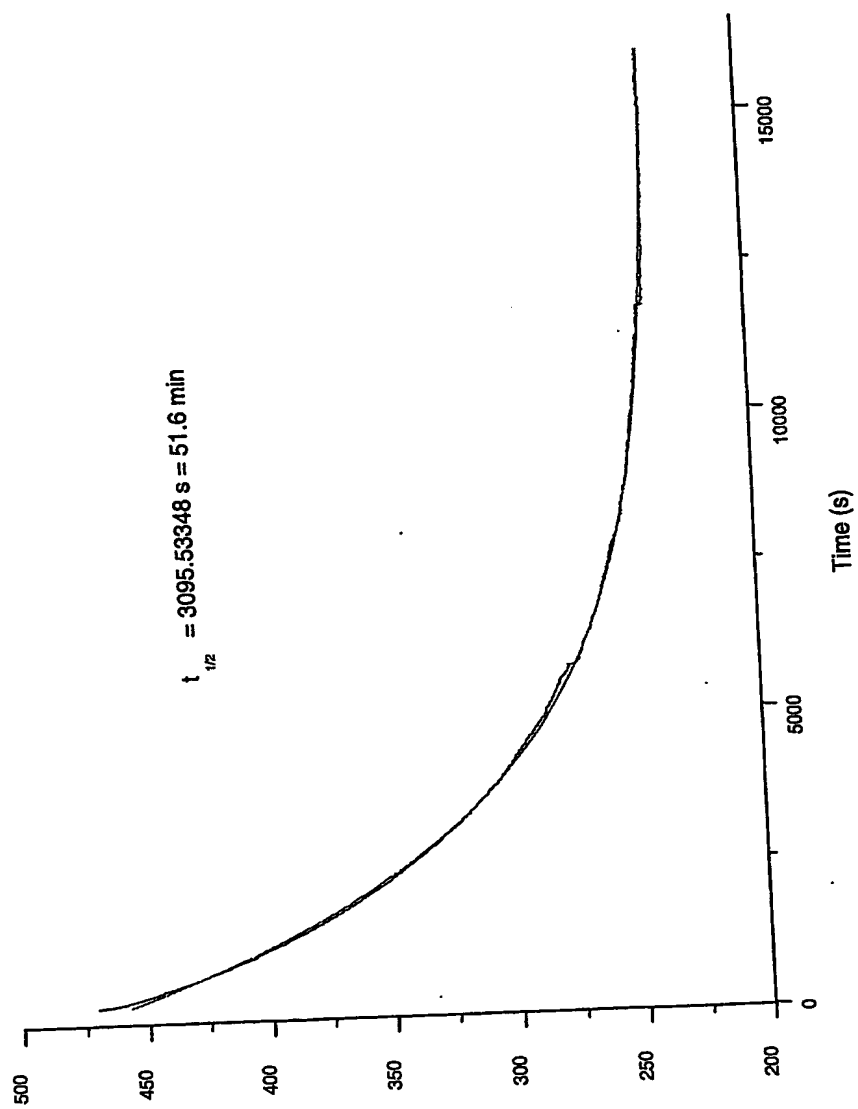


Figure 17a

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 gagcaacaagtggagacttaatgcctcgctggataaatcatcaggacgtagtactttatacattgcagcttctcagcctgg
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 tattgttcacccgtatatccagaaccctgaccctgccgtgtaccagctgagagactctaaatccagtgacaagtctgtctgcct
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 aggtctatggacttcaagagcaacagtgtgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaacaaca
 gcattattccagaagacaccttcttccccagcccagaaagttcctaa

Figure 17b

atgggtgtcactcagacccccaaaattccaggtcctgaagacaggacagagcatgacactgcagtggtcccaggatatgaa
 ccatgaatacatgtcctggtatcgacaagaccaggcatggggctgaggctgattcattactcagttggtgctggtatcactg
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 cagccgcctgagggtctcggccaccttctggcaggacccccgcaaccacttccgctgtcaagtccagttctacgggtctc
 ggagaatgacgagtggaccaggatagggccaaaccgctacccagatcgtcagcgccgaggcctggggtagagcag
 actaa

Figure 18a

MQEXTQIPAALSVPEGENLVLNCSFTDSAIIYNLQWFRQ
 DPGKGLTSLLLIQSSQREQTSGRLNASLDKSSGRSTLYI
 AASQPGDSATYLC AVRPTSGGSYIPTFGRGTS LIVHPYI
 QNPDP AVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDS
 DVIYITDKCVLDMRSMDFKSNSA VAWSNKSDFA CANAF
 NNSIIPEDTFFPSP ESS Stop

Figure 18b

MGVTQTPKFQVLKTGQSM TLQCAQDMNHEYMSWYRQ
 DPGMet GLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDF
 PLRLLSAAPSQTSVYFCASSYVGNTGELFFGEGSRLTVL
 EDLKNVFPPEVA VFEPSEAEISHTQKATLVCLATGFYPD
 HVELSWWWNGKEVHSGVCTDPQPLKEQPALNDSRYAL
 SSR LRV SATFWQDPRNHFR CQVQFYGLSENDEWTQDR
 AKPVTQIVSAEAWGRAD Stop

Figure 19

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1  TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTAATGTG AGTTAGCTCA
   AAGGACCGGA AAACGACCGG AAAACGAGTG TACATTACAC TCAATCGAGT
51  CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC TCGTATGTTG
   GAGTAATCCG TGGGGTCCGA AATGTGAAAT ACGAAGGCCG AGCATAACAAC
101  TGTGGAATTG TGAGCGGATA ACAATTTTAC ACAGGAAACA GCTATGACCA
   ACACCTTAAC ACTCGCCTAT TGTAAAGTG TGTCTTTGT CGATACTGGT
151  TGATTACGCC AAGCTACCTA CTTAAGTATT CTATTTCAAG GAGACAGTCA
   ACTAATGCGG TPCGATGCAT GAATTCATAA GATAAAGTTC CTCTGTCACT
      K Y L L P T A A A G L L L L A
201  TAATGAAATA CCTATTGCCT ACGGCAGCCG CTGGATTGTT ATTACTCGCG
   ATTACTTTAT GGATAACGGA TGCCGTCGCG GACCTAACAA TAATGAGCGC
      NcoI PstI
      -----
251  A Q P A M A K Q E V T Q I P A A L .
   GCCCAGCCGG CCATGGCCAA ACAGGAGGTG ACGCAGATTCT CTGCAGCTCT
   CGGGTCGGCC GGTACCGGTT TGTCTCCAC TGCCTCTAAG GACGTCGAGA
      PstI
      -----
301  . S V P E G E N L V L N C S F T D S .
   GAGTGTCCCA GAAGGAGAAA ACTTGGTTCT CAACTGCAGT TTCACTGATA
   CTCACAGGGT CTTCTCTCTT TGAACCAAGA GTTGACGTCA AAGTGACTAT
   . A I Y N L Q W F R Q D P G K G L
351  GCGCTATTTA CAACCTCCAG TGGTTTAGGC AGGACCCTGG GAAAGTCTC
   CGCGATAAAT GTTGGAGGTC ACCAAATCCG TCCTGGGACC CTTTCCAGAG
   T S L L L I Q S S Q R E Q T S G R .
401  ACATCTCTGT TGTATTTC A GTCAAGTCAG AGAGAGCAAA CAAGTGAAG
   TGTAGAGACA ACGAATAAGT CAGTTCAGTC TCTCTCGTTT GTTCACTTC
   . L N A S L D K S S G R S T L Y I A .
451  ACTTAATGCC TCGCTGGATA AATCATCAGG ACGTAGTACT TTATACATTG
   TGAATTACGG AGCGACCTAT TTAGTAGTCC TGCATCATGA AATATGTAAC
   . A S Q P G D S A T Y L C A V R P
501  CAGCTTCTCA GCCTGGTGAC TCAGCCACCT ACCTCTGTGC TGTGAGGCC
   GTCGAAGAGT CGGACCACTG AGTCGGTGGA TGGAGACACG ACACTCCGGG
   T S G G S Y I P T F G R G T S L I .
551  ACATCAGGAG GAAGCTACAT ACCTACATTT GGAAGAGGAA CCAGCCTTAT
   TGTAGTCCTC CTTGATGTA TGGATGTAAG CCTTCTCCTT GGTGCGAATA
      BamHI
      -----
601  . V H P Y I Q N P D P A V Y Q L R D .
   TGTTCATCCG TATATCCAGA ACCCGGATCC TGCCGTGTAC CAGCTGAGAG
   ACAAGTAGGC ATATAGGTCT TGGGCTTAGG ACGGCACATG GTCGACTCTC
   . S K S S D K S V C L F T D F D S
651  ACTCTAAATC CAGTGACAAG TCTGTCTGCC TATTCACCGA TTTTGATTCT
   TGAGATTTAG GTCACGTGTC AGACAGACGG ATAAGTGGCT AAAACTAAGA
   Q T N V S Q S K D S D V Y I T D K .
701  CAAACAAATG TGTACAAAG TAAGGATTCT GATGTGTATA TCACAGACAA
   GTTTGTTTAC ACAGTGTTC ATTCTAAGA CTACACATAT AGTGTCTGTT
   . C V L D M R S M D F K S N S A V A .
751  ATGTGTGCTA GACATGAGGT CTATGGAATT CAAGAGCAAC AGTGTGTGG
   TACACACGAT CTGTACTCCA GATACCTGAA GTTCTCTGTG TCACGACACC
   . W S N K S D F A C A N A F N N S
801  CCTGGAGCAA CAAATCTGAC TTTGCATGTG CAAACGCCTT CAACAACAGC
   GGACCTCGTT GTTTAGACTG AAACGTACAC GTTTGCGGAA GTTGTGTGTC
      AvrII
      -----
851  I I P E D T F F P S P E S S * *
   ATTATTCAG AAGACACCTT CTTCCTCCAG CCAGAAAGTT CCTAATAACC
   TAATAAGGTC TTCTGTGGAA GAAGGGTTCG GGTCTTTCAA GGATTATTGG
   AvrII EcoRI
      -----
901  TAGGTTAATT AAGAATTCTT TAAGAAGGGG ATATACATAT GAAAAAATTA
   ATCCAATTA TTCTTAAGAA ATTCTTCCC TATATGTATA CTTTTTAAT
      BssHII
      -----
951  L F A I P L V V P F Y S H S A Q A .
   TTATTGCGAA TTCCTTTAGT TGTTCCTTTC TATTCTCACA GCGCGCAGGC
   AATAAGCGTT AAGGAAATCA ACAAGGAAAG ATAAGAGTGT CGCGCGTCCG
   . G V T Q T P K F Q V L K T G Q S M .
1001  TGGTGTCACT CAGACCCCAA AATTCCAGGT CCTGAAGACA GGACAGAGCA
   ACCACAGTGA GTCTGGGGTT TTAAGGTCCA GGACTTCTGT CCTGTCTCGT
      PstI
      -----
1051  . T L Q C A Q D M N H E Y M S W Y
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   ACTGTGACGT CACACGGGTC CTATACTTGG TACTTATGTA CAGGACCATA
   R Q D P G M G L R L I H Y S V G A .
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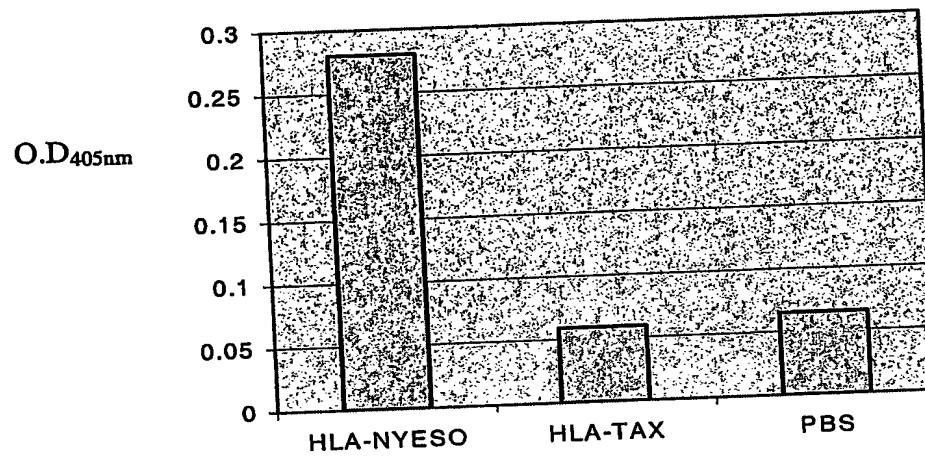
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· G I T D Q G E V P N G Y N V S R S ·
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ACCATAGTGA CTGGTTCCTC TTCAGGGGTT ACCGATGTTA CAGAGGTCTA
· T T E D F P L R L L S A A P S Q ·
1201 CAACCACAGA GGATTTCCTG CTCAGGCTGC TGTGGGCTGC TCCCTCCCAG
GTTGGTGTCT CCTAAAGGGC GAGTCCGACG ACAGCCGACG AGGGAGGGTC
T S V Y F C A S S Y V G N T G E L ·
1251 ACATCTGTGT ACTTCTGTGC CAGCAGTTAC GTCGGGAACA CCGGGGAGCT
TGTAGACACA TGAAGACACG GTCGTCAATG CAGCCCTTGT GGCCCTCGA
· F F G E G S R L T V L E D L K N V ·
1301 GTTTTGTGGA GAAGGCTCTA GGCTGACCGT ACTGGAGGAC CTGAAAAACG
CAAAAAACCT CTCCGAGAT CCGACTGGCA TGACCTCCTG GACTTTTTCG
AvaI BglII
~~~~~
· F P P E V A V F E P S E A E I S ·
1351 TGTTCACCAC CGAGGTCGCT GTGTTTGAGC CATCAGAAGC AGAGATCTCC
ACAAGGGTGG GCTCCAGCGA CACAACTCG GTAGTCTTCG TCTCTAGAGG
H T Q K A T L V C L A T G F Y P D ·
1401 CACACCCAAA AGGCCACACT GGTGTGCCCTG GCCACAGGCT TCTACCCCGA
GTGTGGGTTT TCCGGTGTGA CCACACGGAC CGGTGTCCGA AGATGGGGCT
ApaLI
~~~~~
· H V E L S W W V N G K E V H S G V ·
1451 CCACGTGGAG CTGAGCTGGT GGGTGAATGG GAAGGAGGTG CACAGTGGGG
GGTGCACCTC GACTCGACCA CCCACTTACC CTTCTCCAC GTGTCACCCC
· C T D P Q P L K E Q P A L N D S ·
1501 TCTGCACAGA CCCGCAGCCC CTCAAGGAGC AGCCCGCCCT CAATGACTCC
AGACGTGTCT GGGCGTCGGG GAGTTCCTCG TCGGGCGGGA GTTACTGAGG
R Y A L S S R L R V S A T F W Q D ·
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TCTATGCGAG ACTCGTCGGC GGACTCCCAG AGCCGGTGGA AGACCGTCCT
· P R N H F R C Q V Q F Y G L S E N ·
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GGGGGCGTTG GTGAAGGCGA CAGTTCAGGT CAAGATGCCG GAGAGCCTCT
· D E W T Q D R A K P V T Q I V S ·
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TACTGCTCAC CTGGGTCCTA TCCCGGTTTG GGCAGTGGGT CTAGCAGTCG
XbaI
~~~~~
NotI
~~~~~
A E A W G R A D A A A
1701 GCCGAGGCCT GGGGTAGAGC AGACGCGGCC GCA
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Figure 20



PCT Application

GB0304636

